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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **330th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, University of Cambridge, on Saturday, 19 June 1954, at 11 a.m., when the following papers were read:*

COMMUNICATIONS

An Oxido-reduction Reaction of α-oxoglutarate Coupled with Direct Transfer of an Amino Group.

By F. A. Holton. (*Molteno Institute, University of Cambridge*)

α-Oxoglutarate is known to undergo two distinct oxido-reduction reactions in biological systems:

(*a*) 2α-Oxoglutarate → succinate + CO_2 + α-hydroxyglutarate (Weil-Malherbe, 1937).

(*b*) 2α-Oxoglutarate + ammonia → succinate + CO_2 + glutamate (Krebs & Cohen, 1939).

This communication describes an oxido-reduction which resembles (*a*) in not requiring ammonia but resembles (*b*) in yielding glutamate as the reduced product.

As already briefly reported (Slater & Holton, 1952, 1954), heart muscle sarcosomes (large granules, mitochondria) and crude preparations of yeast hexokinase together bring about an anaerobic disappearance of α-oxoglutarate in the absence of free ammonia, and the reaction is accompanied by phosphorylation. The following additional information has now been gained about this process: (1) The factor in yeast preparations which promotes the reaction is heat-stable and dialysable. Its action cannot be simulated by catalytic amounts of any of the following substances: diphosphopyridine nucleotide, triphosphopyridine nucleotide, diphosphothiamine, pyridoxal phosphate, coenzyme *A*, DL-6-thioctic acid. (2) The disappearance of two molecules of α-oxoglutarate results in the appearance of one molecule each of glutamate, succinate and carbon dioxide. (3) The yeast preparations did not yield ammonia on boiling or during incubation with sarcosomes. (4) The reaction proceeds simultaneously and almost independently of reaction (*b*), which occurs slowly in the presence of large amounts of ammonia.

These results suggest that the reaction studied consists of a dismutation of α-oxoglutarate in which an amino group is transferred directly from some compound in the yeast extract to the oxido-reduction system. The overall process could be expressed by equation (*c*), where $Y\text{-}NH_2$ represents an unidentified amino compound present in the extract.

(*c*) 2α-Oxoglutarate + $Y\text{-}NH_2$ → succinate + CO_2 + glutamate + Y-OH.

This type of reaction, in which an amino donor is converted to a hydroxy compound, instead of a keto compound as in the classical transamination reaction, has been demonstrated explicitly in an anaerobic reaction between L-aspartate and α-oxoglutarate catalysed by sarcosomes. L-Malate and not oxaloacetate was a major product. None of the following amino compounds reacted with α-oxoglutarate under similar conditions: glycine, DL-alanine, L-valine, L-leucine, DL-serine, D-aspartic acid, L-asparagine.

The detailed mechanism of reaction (*c*) may involve a hydrolytic transaminase, or a coupled reaction between a typical transaminase and a dehydrogenase. This coupling would be similar to that suggested by Braunstein (1947) for the mode of action of L-amino acid oxidase.

This work was carried out on behalf of the Agricultural Research Council. My thanks are due to Dr E. L. R. Stokstad of Lederle Laboratories for a gift of DL-6-Thioctic acid.

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Mechanism of Inhibition of Succinic Dehydrogenase by Fluoride and Phosphate.

By E. C. Slater. (*Molteno Institute, University of Cambridge*)

Bonner (1951) showed that inhibition of the succinic oxidase system by fluoride occurred only in the presence of inorganic phosphate. In a detailed study of this inhibition, it was found that both fluoride and phosphate separately, but especially the two together, inhibited succinic dehydrogenase competitively with respect to succinate (Slater & Bonner, 1952). The mechanism of the inhibition was described by equations (1) to (3) (E = succinic dehydrogenase, P = phosphate,

F = fluoride), and the various inhibition constants were calculated.

$$E+P \rightleftharpoons EP, \quad (1)$$
$$E+F \rightleftharpoons EF, \quad (2)$$
$$EP+F \rightleftharpoons EPF \rightleftharpoons EF+P. \quad (3)$$

An alternative mechanism which would fit the kinetic measurements just as well is described by equations (4) and (5):

$$P+F \rightleftharpoons PF, \quad (4)$$
$$E+PF \rightleftharpoons EPF. \quad (5)$$

A choice between the two mechanisms can be made by comparing the inhibitory action of monofluorophosphate with that of fluoride + phosphate. The rates of O_2 uptake (μl./hr.) with various additions were as follows (the figures in brackets are the percentages of inhibition): no addition, 112; + phosphate (0·1 M), 81 (28 %); + fluoride (0·01 M), 103 (8 %); + phosphate + fluoride, 40 (64 %); + fluorophosphate (0·01 M), 86 (23 %). All flasks contained phosphate, 0·002 M; tris(hydroxymethyl)-aminomethane, pH 7·4, 0·06 M; sodium succinate, 0·024 M; ethylenediaminetetraacetate, 0·001 M; heart-muscle preparation, 0·02 ml.; reaction vol., 1 ml.; temperature, 25°.

It is clear that fluorophosphate is much less inhibitory than fluoride + phosphate, in agreement with the mechanism postulated previously.

This work was carried out on behalf of the Agricultural Research Council. I am indebted to Dr Wayne E. White, Ozark-Mahoning Co., Tulsa 1, Oklahoma, U.S.A., for a gift of disodium monofluorophosphate.

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The Resynthesis of Glycogen by Guinea Pig Cerebral Cortex Slices. By F. N. LeBaron.* (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, Maudsley Hospital, London, S.E. 5*)

Brain glycogen has been shown to fall rapidly to a negligible level after death (Kerr, 1936; Chance & Yaxley, 1950). Using the experimental conditions previously found suitable for resynthesis of labile phosphates (McIlwain & Gore, 1951), it has been demonstrated that glycogen is also resynthesized by guinea pig cerebral cortex slices.

Tissue slices were prepared and incubated by methods previously described (McIlwain & Gore, 1951). Variation of the time between death and final equilibration from 10 to 30 min. did not significantly affect glycogen resynthesis. Respiration was measured in Warburg apparatus during incubation. Experiments were terminated by transferring slices immediately into ethanolic KOH, and glycogen was determined by a micromodification of Kerr's method (Kerr, 1936). This consisted of dissolving the tissue in hot alcoholic KOH, extracting cerebrosides from the residue with a chloroform–methanol mixture, hydrolysing the residual solid for 2 hr. in N-H_2SO_4, and determining reducing substances by a micromodification of Nelson's colorimetric method (Nelson, 1944). By this means the following amounts of glycogen expressed as glucose equivalents were found: slices not incubated, 0·4 μmole/g. fresh tissue; incubated 1 hr., 1·1 μmole/g. fresh tissue; incubated 2 hr., 2·3 μmole/g. fresh tissue; incubated 4 hr., 3·0 μmole/g. fresh tissue. There is no appreciable increase between 4 and 6 hr. The variation between animals is about $\pm$ 30 %. Transfer to ethanolic KOH at termination of each experiment required 5–10 sec. No decrease occurred if transfer was delayed up to 1 min. or more. The following facts have been demonstrated as evidence that the substance synthesized is, in fact, glycogen: (1) A red-purple colour, similar to that given by glycogen is obtained with dilute Lugol's iodine solution from the substance isolated as glycogen, but from incubated slices only. (2) This substance gives colour with anthrone (Kahan, 1953) before hydrolysis, as do polysaccharides. (3) This substance does not have reducing action as measured by Nelson's method (Nelson, 1944) before hydrolysis, but the reducing substance obtained on hydrolysis is at least 90 % fermentable by yeast.

Variations in and additions to the incubating medium have shown that glycogen synthesis proceeds equally well in Krebs-Ringer with phosphate buffer or in phosphate-free medium with 'THAM' buffer. Addition of insulin to the normal Krebs-Ringer phosphate to the amount of 0·3 unit/ml. has no effect on the rate or amount of glycogen synthesis. A graded inhibition of synthesis with increasing concentrations of 2:4-dinitrophenol is obtained.

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γ-Methylene-α-Oxoglutaric acid: a Constituent of Groundnut Plants (*Arachis hypogaea*).

By L. Fowden and J. A. Webb. (*Department of Botany, University College, London, W.C.* 1)

Following the isolation of γ-methyleneglutamic acid (MGA) from groundnut plants (Done & Fowden, 1952), evidence has now been obtained that the keto-acid analogue, γ-methylene-α-oxoglutaric acid (MOG) is a normal constituent of the plants. Towers & Steward (1954) have obtained evidence indicating its presence in tulips.

The keto-acids present in 6-day-old groundnut seedlings were investigated by paper chromatography of their 2:4-dinitrophenylhydrazine (DNPH) derivatives using *n*-butanol saturated with 6% (w/v) NH_3 solution as developing solvent. In addition to spots corresponding to the derivatives of α-oxoglutaric (OG), pyruvic and glyoxylic acids, an additional spot, moving just in advance of that of OG, was present on chromatograms. The position of this spot indicated that it was probably due to a dicarboxylic keto-acid of higher mol. wt. than OG. It was therefore co-chromatographed with the DNPH derivative of MOG prepared enzymically by transamination from MGA and OG (following Fowden & Done, 1953); no separation between the two substances occurred during a 40 hr. development.

A larger scale isolation of the DNPH derivative of the new keto-acid was made from one kg. fresh weight of seedlings, using a column packed with Whatman cellulose powder to separate the hydrazones. About 7 mg. of the derivative resulted. 4 mg. of the DNPH derivative of MOG was obtained from a larger-scale transamination, the derivative of unchanged OG again being separated on cellulose powder. The isolated and biologically synthesized products were shown to have similar absorption spectra when dissolved in N-NaOH (differing from that of the OG derivative), and to be essentially identical by X-ray powder photography of their ammonium salts. On hydrogenation (Towers, Thompson & Steward, 1954), both yielded an amino acid inseparable chromatographically from γ-methylglutamic acid in five developing solvents. Chemical synthesis would facilitate an absolute characterization of the isolated material.

Three additional unknown keto-acids, all moving more slowly than OG when chromatographed as their DNPH derivatives, have been encountered in the various groundnut tissue extracts examined.

We wish to thank Prof. K. Lonsdale, F.R.S., for X-ray comparisons and Prof. F. C. Steward for supplying papers in manuscript form. One of us (J. A. W.) wishes to thank the Agricultural Research Council for a personal maintenance grant.

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The Silver Thiol Electrode and Ion-exchange Reactions on the Electrode Surface.

By R. Cecil. (*Department of Biochemistry, University of Oxford*)

During recent work on the potentiometric titration of thiols with silver nitrate (Cecil & McPhee, to be published), it was found that, in neutral or alkaline solution, the silver electrodes acquired a reversibility to thiol. This suggested that the thiols react to form a layer of silver mercaptide on the electrode surface. The production of silver thiol electrodes in this way depends on rather critical silver-plating conditions, and a better method is to dip the freshly plated electrodes in aqueous H_2S for a few seconds and allow the Ag/Ag_2S electrodes thus formed to 'exchange' with the thiol.

The occurrence of ion-exchange reactions on the surface of silver electrodes was confirmed by experiments with other anions. Thus starting with Ag/AgCl, electrodes were made which were in turn reversible to Br^-, CNS^-, CrO_4^{2-}, CO_3^{2-}, I^-. In the same way Ag/Ag_2S electrodes, prepared as described, were made reversible to I^-, Br^- and finally Cl^-. All these electrodes are, of course, reversible to Ag^+.

The reaction at the electrode surface can be written alternatively:

$$RSH + Ag \rightleftharpoons RSAg + H^+ + e,$$
$$RS^- + Ag \rightleftharpoons RSAg + e.$$

One expression of the potential is

$$E = E_0 - \frac{RT}{F} \ln RSH + \frac{RT}{F} \ln H^+,$$

where RSH represents the concentration of unionized thiol. Since this depends on the thiol pK and on pH, measurement of E can give information about the ionization of the thiol group and shows that it ionises before the amino group.

When Dixon & Quastel (1923) and later Michaelis & Flexner (1928) investigated the oxidation-reduction potential of the cystine-cysteine system, they

found that the potentials they obtained bore the same relationship to thiol and hydrogen-ion concentration. This suggests that their electrodes, of Pt, Au and Hg, had reacted with the cysteine and were not inert (see also Freedman & Corwin, 1949).

Detailed studies have so far been made with electrodes reversible to thioglycollic acid, cysteine and homocysteine. The thioglycollic electrodes behave as predicted, but the cysteine, and to a lesser extent the homocysteine, electrodes show anomalous responses at high pH. These correspond approximately to the transfer of half an electron per molecule of cysteine. The explanation is still being sought, but is likely to be some form of association between cysteine molecules.

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The Occurrence of a Methylsulphonium Derivative of Methionine (α-Aminodimethyl-γ-butyrothetin) in Asparagus. By F. CHALLENGER and BARBARA J. HAYWARD. (*Department of Organic Chemistry, The University, Leeds* 2)

The isolation of dimethyl-β-propiothetin chloride $Me_2S^+(Cl^-).CH_2.CH_2.COOH$ from *Polysiphonia fastigiata* by Challenger & Simpson (1948) of the corresponding picrate from *Enteromorpha intestinalis* (Bywood & Challenger, 1953) and of the platinichloride from *Spongomorpha arcta* (Bywood, 1953), established the natural occurrence of sulphonium compounds. An unidentified dimethylsulphonium compound (or compounds) was detected in asparagus (Bywood, Challenger, Leaver & Whitaker, 1951). Jansen (1948) isolated 1:3-dithio*iso*butyric $HS.CH_2.CH(COOH).CH_2.SH$ as the disulphide from asparagus; it did not produce methanethiol in the urine after ingestion.

The sulphonium salt in asparagus differed from the algal thetin; alkaline decomposition of asparagus extracts to dimethyl sulphide required long boiling while the seaweed thetin was readily decomposed by alkali.

An ethanolic extract of asparagus tips was evaporated at 40° under diminished pressure, the residue dissolved in water and, after removal of protein and chlorophyll, applied to a column of sulphonated cross-linked polystyrene (PSX). The retained sulphonium compound was then eluted with ammonia, together with basic amino acids and the eluate applied to 'Deacidite FF' to retain amino acids. The sulphonium effluent, when concentrated, gave a chloroplatinate m.p. 190–193° which evolved dimethyl sulphide with boiling alkali. It had mixed m.p. 196° with the synthetic chloroplatinate m.p. 201°. Both chloroplatinates, after decomposition with metallic silver by the method of Dudley (1929), gave picrates m.p. 158° (synthetic) and 153° (asparagus). The mixed m.p. was 156°. Further purification of the small amount available was impossible. The picrate of the asparagus product dissolved in hydrochloric acid was repeatedly extracted with ether and the resulting chloride solution concentrated. Paper chromatography (butanol–acetic acid–water–ninhydrin) gave a spot corresponding to that obtained with authentic methylmethioninesulphonium iodide and a very faint spot, possibly due to lysine. The solution was then boiled with alkali until evolution of dimethyl sulphide ceased.

The authentic sulphonium iodide was similarly decomposed. Both solutions were passed through PSX, eluted with ammonia and concentrated. Paper chromatography as before gave two pairs of similar spots corresponding to methionine sulphoxide (arising by oxidation of methionine) and homoserine. Asparagus would therefore appear to contain a salt of α-aminodimethyl-γ-butyrothetin, $Me_2S^+(X^-).CH_2.CH_2.CH(NH_2).COOH$.

McRorie *et al.* (1954) isolated methylmethioninesulphonium bromide from cabbage and detected it in parsley, turnip greens, turnips, pepper, onion and lettuce; aqueous hydrolyses of the natural and synthetic products gave homoserine. Formation of dimethyl sulphide is also mentioned.

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The Alkaline Degradation of the Human Blood-group Substances. By K. W. KNOX and W. T. J. MORGAN. (*Lister Institute, London, S.W.* 1)

Treatment of the human *A*, *B* and *H* blood-group mucoids with dilute Na_2CO_3 (pH 10·8 at 100°) results in decrease in viscosity, loss of serological activity, formation of a chromogen from some of the *N*-acetylhexosamine constituents and to complete disintegration of the mucoid (Morgan, 1946). Dialysis of the alkali-treated substances yields a diffusate which gives an immediate colour with Ehrlich's reagent (Aminoff, Morgan & Watkins, 1952), whereas any indiffusible material remaining fails to give this colour reaction.

More gentle treatment ($BaCO_3$ pH 8·5 at 100°) of *N*-acetylglucosamine gives a maximum colour in about 48 hr. Human group substances give in 8 hr. about 15 % of diffusible material, which shows some serological activity and contains a peptide moiety and mucopolysaccharides. The latter possess all the component sugars (L-fucose, D-galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine) of the original substance. The indiffusible material shows some loss in serological activity and viscosity but otherwise is little changed. To reduce the amount of decomposition which occurs when the split products are subject to prolonged heating at pH 8·5, the material after each 1 hr. period at 100° is dialysed and the diffusible material removed. After 32 hr. heating, 10 % of the mucoid remains indiffusible. This material shows little serological activity but largely retains the composition of the original material. The diffusate is concentrated and fractionated at 50, 65, 80 and 90 % (v/v) levels of ethanol. The addition of ether gives a final fraction. About a third of the diffusible product remains soluble.

The ethanol fractions are of the same general composition as the starting material, but all activity is in the fractions precipitated below 65 % ethanol. The ether-soluble fraction contains a series of oligosaccharides which possess the chromogen. These materials have R_F values in collidine of 0·11, 0·22, 0·34 and 0·53 (R_F, *N*-acetylglucosamine, 0·50; *N*-acetylglucosamine chromogen, 0·78).

Monosaccharides and disaccharides, which possess 1:1, 1:3, 1:4 and 1:6 linkages, were treated at 100° and pH 8·5 and the changes studied.

Chromogen is formed from *N*-acetylglucosamine at pH 5·4 and a maximum Ehrlich colour value, equivalent to 70 % of that obtained after 4 min. heating at pH 10·8, was found after 24 hr. at 100°. The group substances behave similarly but the maximum colour (6 days at 100°) is only 10 % of the value obtained from the material after 12 min. at pH 10·8 and 100°.

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Disulphide Interchange Reactions. By A. P. RYLE and F. SANGER. (*Biochemical Laboratory, University of Cambridge*)

The disulphide interchange reaction first reported by Sanger (1953) has been studied in acid, neutral and slightly alkaline solutions using various model systems, with a view to finding under what conditions the reaction occurs and how it may be prevented during chemical studies on protein structure.

In acid solution the reaction between cystine and *bis*-DNP-cystine was followed spectrophotometrically after removal of the unchanged *bis*-DNP-cystine by ether extraction. The yellow ether-insoluble product formed has been crystallized and identified as *mono*-*N*-DNP-cystine; it is formed by the following overall reaction:

$$\text{DNP-CyS.SCy-DNP} + \text{CyS.SCy} \rightleftharpoons 2\text{DNP-CyS.SCy}.$$

The rate of the reaction is maximal in HCl stronger than 9 N, and in such acid at 37° the reaction between 10^{-3} M cystine and 10^{-4} M *bis*-DNP-cystine stops in 10 hr., when about 90 % of the *bis*-DNP-cystine has reacted. Half the reaction occurs in about 1·5 hr. In 7 N-HCl the reaction is slow, and in 5 N-HCl negligible. The reaction in 10 N-HCl is markedly inhibited by the addition of cysteine to give a ratio SH:SS = 0·01, and is slower in H_2SO_4 than in HCl.

In a mixture of 10 N-H_2SO_4–glacial acetic acid (3:1 v/v) at 37° very little reaction occurs in 13 days. Conditions such as these have been used to effect partial hydrolysis of fragments of insulin without disulphide interchange occurring (see following abstract).

The reaction in neutral solution proceeds rather more slowly and was studied chiefly with a system of 10^{-3} M *bis*-DNP-cystine and 10^{-3} M cystinyl-*bis*-glycine in 0·017 M phosphate buffer. The peptide was used in place of cystine because of the low solubility of the latter at pH 7. It was found that the reaction was about eight times as rapid when oxygen was excluded than when it was present, that the presence of 10^{-4} M cysteine or glutathione produced a very great acceleration and that *p*-chloromercuribenzoate or *N*-ethyl maleimide caused complete

inhibition. The latter inhibitor has been used in structural studies to inhibit the interchange reaction of cystine peptides in neutral solutions.

These results taken together suggest that the reaction in neutral solution is mediated by RS' ions formed by dissociation of thiols (Bersin & Steudel, 1938), which in turn arise from hydrolytic fission of the disulphide bond (Cecil, 1950).

$$R_1SSR_1 + OH^- \rightleftharpoons R_1S^- + R_1SOH,$$
$$R_2SSR_2 + R_1S^- \rightleftharpoons R_1SSR_2 + R_2S^-, \quad \text{etc.}$$

This conclusion is supported by the finding that the speed of the reaction between *bis*-DNP-cystine and glutathione (disulphide) increases with increasing pH. The reaction in acid solution must proceed by some other mechanism since it is inhibited, not catalysed, by thiols.

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The Disulphide Bridges of Insulin. By F. Sanger, L. F. Smith and Ruth Kitai. (*Department of Biochemistry, University of Cambridge*)

The two polypeptide chains of insulin contained in a molecule of mol. wt. 6000 (Harfenist & Craig, 1952) are joined together by the disulphide bridges of three cystine residues. After breaking these bridges by oxidation, the chains could be separated and their amino acid sequences determined (Sanger & Tuppy, 1951; Sanger & Thompson, 1953). They may be represented as follows:

(*A*) Gly ~ Glu . $CySO_3H$. $CySO_3H$. Ala . Ser . Val .
(1) (5) (6) (7) (8) (9) (10)

$CySO_3H$. Ser ~ Tyr . $CySO_3H$. Asp,
(11) (12) (19) (20) (21)

(*B*) Phe ~ His . Leu . $CySO_3H$. Gly ~ Leu . Val .
(1) (5) (6) (7) (8) (17) (18)

$CySO_3H$. Gly . Glu . Arg . Gly . Phe ~ Ala.
(19) (20) (21) (22) (23) (24) (30)

To determine the position of the disulphide bridges, intact insulin was treated with chymotrypsin and the hydrolysate concentrated and brought to pH 5. This yielded a precipitate ('chymotrypsin core') and a solution. The soluble material was fractionated by ionophoresis on paper at pH 6 (Michl, 1951), using the cyanide nitroprusside test (Toennies & Kolb, 1951) to locate the cystine-containing peptides. Each fraction was then oxidized to the corresponding cysteic acid-containing peptides, which were fractionated by ionophoresis, detected by the ninhydrin reaction, eluted and hydrolysed, to identify the amino acids present. One cystine-containing peptide gave rise on oxidation to $CySO_3H$. Asp . NH_2(*A*c1) and Leu . Val . $CySO_3H$. Gly . Glu . Arg . Gly . Phe . Phe(*B*c4). It therefore has the structure

Leu . Val . Cy . Gly . Glu . Arg . Gly . Phe . Phe
|
S
|
S
|
Cy Asp . NH_2

and establishes the presence of a disulphide bridge between positions *A* 20 and *B* 19 in insulin. Other similar peptides from this and from a peptic hydrolysate confirmed the presence of this bridge.

The remaining two cystine residues were present in the 'chymotryptic core' and in a similar preparation from a peptic hydrolysate. These cores were subjected to acid hydrolysis under conditions where the 'interchange reaction' is minimal (100 mg. 'core', 5 ml. 20N-H_2SO_4, 3 ml. glacial acetic acid, 2 ml. water, 3 μl. thioglycollic acid. 100°. 30–60 min.). After removal of the H_2SO_4 with an ion-exchange resin, the hydrolysate was fractionated by ionophoresis at pH 6 and 3·6. The cystine-containing bands were eluted, oxidized and subjected to ionophoresis at pH 3·6, to separate the cysteic acid peptides from other non-cysteic acid peptides, and also to some extent from one another. In this manner the following cystine peptides were identified:

His . Leu . Cy
|
S
|
S
|
Cy . Ala

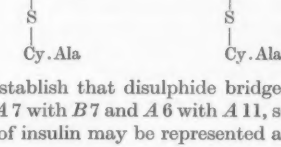
Cy . Gly
|
S
|
Cy . Ala

Ser . Val . Cy
|
S
|
S
|
Gln . Cy

Leu . Cy
|
S
|
Cy . Ala

Val . Cy
|
S
|
S
|
Glu . Cy

These establish that disulphide bridges connect positions *A* 7 with *B* 7 and *A* 6 with *A* 11, so that the structure of insulin may be represented as follows:



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The Partial Vitamin E Activity of Certain Redox Dyes. By T. Moore, I. M. Sharman and R. J. Ward. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

In previous work (Moore, Sharman & Ward, 1953*a*, *b*, *c*) we followed up reports by Dam, Prange & Sondergaard (1952) that various natural and artificial redox substances, including methylene blue, possess some degree of vitamin E activity. Under our experimental conditions we failed to confirm that methylene blue prevented resorption gestations in deficient rats, or the haemolysis of their erythrocytes by dialuric acid. It was also ineffective in preventing degeneration of the testes. Full activity was found, however, against brown discoloration of the uterus and kidney lesions in rats kept for long periods on diets deficient in vitamin E. Using protection against brown uterus as the criterion we confirmed that thiodiphenylamine was also active, and extended the list to include Bindschedler's green, malachite green, leuco-malachite green and new methylene blue. Several other redox dyes were found inactive at the levels tested. No activity was found for ascorbic acid or cystine, or for any other substance with redox properties but not in the dyestuff class.

In further experiments we have now observed that brown uterus is prevented by rosaniline, as 0·1% of the diet and by methyl violet (0·126%). No protection was given by *p*-aminobenzoic acid (0·1%) or by sulphapyridine (0·1%). None of these substances was effective in the haemolysis test.

In trials with graded levels of methylene blue, 0·032% was found sufficient for protection against brown uterus. This level is only one-quarter of the dose (0·126%) we previously found to be ineffective in preventing resorption gestations or degeneration of the testes. Methylene blue was also tested for its ability to prevent the decline of weight in rats that were beginning to show signs of muscular dystrophy after being kept for nearly a year on the deficient diet. Substantial increases in weight were observed in rats dosed either with the dye or with (±)-α-tocopheryl acetate. The theory that some redox dyes have the anti-dystrophic action of vitamin E, but without its power to protect the reproductive functions, is under investigation.

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The Metabolism of Propionic Acid by Sheep-rumen Epithelial Tissue. By R. J. Pennington and T. M. Sutherland. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

It has been shown previously that propionic acid is metabolized *in vitro* by sections of sheep-rumen epithelium, and that the rate of uptake of this acid is increased by the presence of carbon dioxide. It was accordingly suggested that propionic acid (or a derivative) may be carboxylated by addition of carbon dioxide. On the basis of other evidence it was considered likely that the carboxylation step did not follow on pyruvate formation (Pennington, 1954).

Propionic acid, when metabolized together with pyruvic acid, completely suppressed the formation of ketone bodies from the latter. The quantity of pyruvate metabolized was correspondingly decreased. In this respect the 'antiketogenic' action of propionate in this ruminant tissue appears to differ from that of glucose, previously described (Pennington & Sutherland, 1953). Propionate similarly suppressed ketone body formation from lactate.

Lactic acid is produced when propionate is metabolized alone. The quantities formed can account for 30–50% of the propionate disappearing.

When propionate was metabolized in the presence of $^{14}CO_2$ the isotope was incorporated into tissue

metabolites. Approximately one molecule of CO_2 was 'fixed' for each three molecules of propionate metabolized. Most of the isotope was located in the —COOH of the lactate formed. Less than one-tenth as much CO_2 was fixed during the metabolism of pyruvate as when an equivalent quantity of propionate was metabolized.

When $CH_3CH_2{}^{14}COOH$ was metabolized the greater part of the isotope appeared in the carbon dioxide. The specific activity of the lactate formed was only about one-fifth of that of the propionate; this is apparently not due to dilution by endogenously produced lactate because lactate production by the tissue in the absence of substrate is low.

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The Association of Insulin with the Reticulin of Rat Spleen. By J. H. Ottaway. (*Department of Biochemistry, University College, London*)

Chayen & Smith (1954) have shown that insulin labelled with fluorescein becomes bound to the connective tissue of several organs when it is injected into rats. It was of interest to find out whether endogenous insulin is similarly bound to connective tissue fibres.

Reticulin can be conveniently prepared from rat spleen by a method suggested by Dr H. Fell. The spleen is cut freehand into thin slices, which are washed to remove adhering blood, and shaken in Krebs-Ringer phosphate solution at 37° until the tissue has disintegrated. The process may be hastened if necessary by gentle trituration in a mortar. The reticulin fibres are separated by decantation and shaken with successive portions of Ringer solution until the solution is free from haemoglobin. About 50 mg. of moist white fibres, contaminated by only a small number of adhering cells, are obtained from one spleen. The preparation takes 60–90 min., using about 250 ml. of Ringer solution.

In the experiments to be described, spleens were taken from fasted rats which had been injected with heparin to facilitate removal of blood from the tissue. Insulin was detected by the rat diaphragm technique, using the conditions described by Ottaway (1953). The glucose uptake of quarter-diaphragms (weight about 50 mg.) showed a significant increase over the control rate during 45 min. incubation with 10 mg. reticulin from normal spleen. Incubation with reticulin from spleens of alloxan-diabetic rats did not affect the glucose uptake. The solution used for the final (10 min.) washing of the reticulin fibres did not contain any detectable amount of insulin.

It is concluded that the connective tissue of rat spleen (and presumably of other organs also) normally contains insulin which can slowly dissociate in an active form. The relations of this phenomenon to the binding of insulin in tissues described by Stadie and his collaborators (Stadie, 1954), and to the insulin-like effect of growth hormone (Ottaway, 1953), have been studied.

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The Association of Insulin with Reticulin in Rat Tissues. By J. Chayen and R. H. Smith. (*Wheatstone Laboratory, King's College, London, W.C. 2 and School of Biochemistry, University of Cambridge*)

Glucose utilization studies on the isolated rat diaphragm after exposure to insulin led Stadie (1954) to conclude that insulin is bound to certain, unspecified tissue elements. Subsequent experiments (Stadie, 1954) with isotopically labelled insulin supported this conclusion but did not indicate the site at which the insulin was bound.

In an attempt to elucidate the site of action of insulin in isolated tissues, we observed that conjugates of insulin with fluorescent dyes possess a high affinity for the reticulin components of rat diaphragm. The insulin conjugates were prepared with either dimethylaminonaphthalene-5-sulphonyl chloride (Weber, 1952) or fluorescein isocyanate (Kaplan & Coons, 1951); they contained 2 moles of dye per mole of insulin. Insulin thus modified, retained at least 50% of its original hypoglycaemic activity.

The insulin-binding effect was observed in diaphragms taken from rats injected 2 hr. previously with labelled insulin and in normal diaphragms incubated for periods of 15 to 60 min. with Stadie medium containing labelled insulin. Simple teasing of the fresh, unfixed tissue with fine needles in Stadie medium was found to give the best preparations for fluorescence microscopy. In these teased preparations the characteristic yellow fluorescence of the administered insulin conjugate can readily be seen to be associated with the fine reticulin network of the muscle. Tendon collagen fibres, in contrast to reticulin, do not bind insulin conjugates to an observable extent. The reticulin of spleen and adipose tissue preparations also shows a marked affinity for insulin conjugates.

Controls were carried out with the following substances which were administered 2 hr. before excising the diaphragms: fluorescein, the fluorescein conjugates of whole serum (human) lactoglobulin (cryst.), ribonuclease (cryst.), prolactin and somatotrophin (cryst.). Apart from somatotrophin, which appeared to bind like insulin, none of these substances had an observable affinity for rat diaphragm reticulin.

The reversible nature of the binding of insulin by reticulin is indicated by the fact that the yellow fluorescence of reticulin in diaphragm preparations slowly becomes diffuse: after standing 2–3 hr. in Stadie medium, at room temperature, it is not easy to distinguish the yellow secondary fluorescence from the primary fluorescence of the tissue. The experiments of Ottaway (1954) also provide evidence that this binding effect is reversible.

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Dependence of Sulphate Reduction and Oxygen Utilization on a Cytochrome in *Desulphovibrio*.

By J. R. Postgate (introduced by V. A. Knivett). (*Chemical Research Laboratory, Teddington*)

The sulphate-reducing bacteria are unusual among anaerobes because they contain a thermostable cytochrome (α, 553 mμ.; β, 525 mμ.; γ, 419 mμ.). It may be extracted in solution and concentrated; a soluble green protein absorbing at 630 mμ. has none of the properties of a conventional cytochrome (Postgate, 1954).

Cells incubated *in vacuo* with excess of sulphate or sulphite showed α and β bands of diminished intensity compared with cells without sulphate provided precautions were taken to remove sulphide; the 630-component was unaffected. This phenomenon was temporarily antagonized by structural analogues of sulphate (selenate or monofluorophosphate) suggesting that the cytochrome is directly concerned in sulphate reduction.

The cytochrome bands disappear on shaking a cell suspension in air, and return on standing or passing in H_2 (Postgate, 1954), suggesting that oxidation of substrate with oxygen by these bacteria is in principle possible. Washed suspensions of the Hildenborough strain have now been shown to oxidize lactate or pyruvate slowly in air ($-Q_{O_2}$ 8 mm.^{3}/mg. dry wt./hr.; cf. Q_{CO_2} values of 60–120 with sulphate). If gaseous hydrogen was the substrate, however, a rapid Knallgas reaction occurred, and cells rich in hydrogenase reduced oxygen as readily as they reduced sulphate, though not as fast as they reduced methylene blue. The rate of the Knallgas reaction depended on the oxygen concentration up to 4% (v/v); higher oxygen concentrations progressively inhibited the reaction presumably owing to oxidative destruction of hydrogenase during the experiments.

Cetyltrimethylammonium bromide (100 μg./mg. dry wt. cells) liberated cytochrome into the medium and abolished sulphate reduction without affecting the reduction of methylene blue by hydrogen. Such cells conducted the Knallgas reaction only slowly, but the rate of reaction was augmented by addition of a concentrate of the cytochrome, but not by denatured cytochrome or muscle cytochrome *c*. Hence the rate of the H_2–O_2 reaction is dependent on the cytochrome concentration. Both crude and purified preparations of the cytochrome are autoxidizable, and it is likely that the utilization of oxygen, paradoxical in an anaerobe of so exacting a character, is simply a result of this chemical property.

I am indebted to Dr Keilin for suggesting the primary experiments on oxygen utilization.

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The Hydrolysis of Cortisone Acetate by Enzymes of Human Blood. By F. Hobbiger, Sylvia A. Simpson and J. F. Tait. (*The Middlesex Hospital, Medical School, London, W.* 1)

Although Zeller, Fleisher, McNaughton & Schweppe (1949) have described the hydrolysis of deoxycorticosterone acetate by stromata of human red cells, detailed studies of the processes involved in the hydrolysis of steroid acetates by enzymes of human blood have not been reported. This lack of information is probably due to the difficulty of applying the usual techniques for the study of esterases because of the low solubility of most steroid esters in biological fluids.

A technique has been developed for the measurement of the rate of hydrolysis of cortisone acetate at low concentrations. 30 μg. [4-^{14}C]cortisone acetate in 3 ml. solution were incubated with preparations of human red cells and plasma and purified true and pseudo-cholinesterase under anaerobic conditions at 37° and at a pH of 7. The labelled material was extracted by partition methods and the resulting residue chromatographed in the Bush *B* (3) system (Bush, 1952). The steroid ester and alcohol were located by the method of Haines & Drake (1950) and the radioactivity in the appropriate ultraviolet adsorbing areas was measured by mica-window counting. The ratio of the counts due to the alcohol compared with the ester gave the value for the amount of hydrolysis.

Using this technique the hydrolysis of cortisone acetate by human red cells, which contained the main enzymatic activity of whole blood, was investigated in detail. No difference was found between intact and lysed cells. A comparison of the activity of highly purified true cholinesterase and preparations of red cells, in concentrations equally active against acetylcholine, showed that true cholinesterase was only partly responsible for the hydrolysis of cortisone acetate by human red cells. This indicated that in addition to true cholinesterase another esterase or esterases must be involved. This was confirmed by a study of the effect of potent inhibitors at concentrations which completely abolished cholinesterase activity. Thus, although di*iso*propylphosphorofluoridate and tetraethylpyrophosphate inhibited completely the enzymatic hydrolysis of the steroid ester, most of the activity was not affected by eserine or 3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate.

The relation of this esterase to other esterases present in human blood (Mounter & Whittaker, 1953) will be discussed.

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The Incorporation of Halogenated Pyrimidines into Deoxyribonucleic Acids. By D. B. Dunn and J. D. Smith. (*Agricultural Research Council Plant Virus Research Unit, Molteno Institute, Cambridge*)

The growth of *Streptococcus faecalis* R. is inhibited by 5-bromouracil. Weygand, Wacker & Dellweg (1952) demonstrated that after growth in the presence of 5-bromouracil containing the isotope ^{82}Br substantial amounts of labelled 5-bromouracil could be isolated from *S. faecalis* nucleic acids after hydrolysis with $HClO_4$.

When *Bacterium coli* is made thymine-requiring by growth in a medium containing sulphanilamide (Winkler & de Haan, 1948), 5-bromouracil and 5-iodouracil inhibit growth. 2–3 hr. after their addition the bacterial growth rate is decreased and thereafter remains approximately constant. The bacteria grow to their normal maximum density, but the viable count is reduced. The degree of inhibition by 5-bromouracil increases with the ratio 5-bromouracil/thymine in the medium. *Bact. coli* inhibited by 5-bromouracil or 5-iodouracil will support the growth of the bacteriophages T_2r and T_5.

Deoxyribonucleic acids (DNA) from *Bact. coli*, T_2r and T_5 grown in the presence of 5-bromouracil, were hydrolysed in 72% (w/w) aqueous $HClO_4$ (Marshak & Vogel, 1950), and the bases separated by two-dimensional paper chromatography. 5-Bromouracil, identified by paper chromatography, electrophoretic mobility and ultraviolet spectroscopy, was found to replace quantitatively a fraction of the thymine (26% in *Bact. coli* DNA, 77% in T_2r and 30% in T_5 DNA). 5-Iodouracil on hydrolysis in $HClO_4$ in the presence of DNA breaks down quantitatively to uracil. Hydrolysis of DNA from *B. coli* and T_2r grown in the presence of 5-iodouracil gave uracil in amounts corresponding to a replacement by 5-iodouracil of 17% (*Bact. coli*) and 50% (T_2r) of the thymine in the nucleic acid.

After hydrolysis of the deoxyribonucleic acids with deoxyribonuclease and rattlesnake venom diesterase, substances with the expected properties of 5-bromouracil and 5-iodouracil deoxyribonucleo-

tides were isolated. These migrated ahead of the other mononucleotides on paper electrophoresis at pH 9. On hydrolysis in 72 % $HClO_4$, 5-bromouracil deoxyribonucleotide gave equimolar proportions of 5-bromouracil and phosphate, while 5-iodouracil deoxyribonucleotide gave uracil.

Although purified T_2r and T_5 containing 5-bromouracil or 5-iodouracil had the normal ultraviolet absorption spectra, and electron microscopy of the T_2r 'bromouracil' preparations showed largely particles of normal structure, a large proportion of the particles in each of these preparations were non-infective. T_2r preparations in which 77 % of the thymine had been replaced by 5-bromouracil contained about 70 % non-infective particles.

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The Oxidation *in vitro* of Labelled Glucose and Acetate by Slices of Lactating Rat Mammary Tissue. By W. G. Duncombe and R. F. Glascock. (*National Institute for Research in Dairying, University of Reading*)

By a technique exactly similar to that previously described (Duncombe & Glascock, 1953) with sheep tissue the oxidation *in vitro* (bicarbonate-Ringer) of uniformly labelled glucose (0·3 %) and doubly labelled acetate ($^{14}CH_3{}^{13}COONa$; 0·02 M) has now been studied in lactating rat mammary tissue. It oxidizes more carbon, when glucose is sole substrate, than does sheep tissue (rat 5–10, sheep 1–2 μg. C/hr./mg. dry weight tissue). Whereas with sheep mammary tissue acetate increased the oxidation of glucose carbon, in rat mammary tissue acetate has no measurable effect. Pyruvate (0·02 M), however, reduces by about 50 % the oxidation of glucose. This is presumably due to dilution by unlabelled pyruvate of labelled pyruvate derived from glucose via the glycolytic cycle.

Rat mammary tissue oxidizes acetate, as sole substrate, much less actively than does sheep tissue (rat 0·1–0·4, sheep 1–4 μg. C/hr./mg. dry weight tissue) and about 50 % more carboxyl carbon than methyl carbon appears in the CO_2. In 23 experiments (seven animals) the ratio of carboxyl to methyl carbon oxidized to CO_2 was in the range 1·11–1·57 with a mean of 1·42.

Whereas in sheep mammary tissue, glucose increased the amount of acetate carbon oxidized, in rat tissue glucose reduces the amount oxidized by about 50 %, the effect on the two carbon atoms being quantitatively different however. The mean ratio of carboxyl to methyl carbon oxidized then becomes 2·06 with a range of 1·72–2·93; i.e. glucose depresses the amount of methyl carbon oxidized more than it does carboxyl carbon. It would thus appear that the observed effect of glucose on the oxidation of acetate is not due solely to the production of unlabelled C_2 units which dilute those derived from the unlabelled acetate.

It will be noted that the effect of added glucose on the oxidation of acetate by rat mammary tissue is different from that reported by Popjak & Tietz (1954). Using phosphate saline (gas phase = O_2) they found that rat mammary tissue oxidized about 5 times more acetate carbon in the presence of glucose than in its absence. We have repeated their experiment using phosphate saline and have observed the same effect. There is thus a marked difference in the metabolism of the slices in the two media.

Thanks are due to Dr S. J. Folley, F.R.S., for his advice and encouragement in this work, to Dr D. H. Tomlin for carrying out the ^{13}C determinations in the Agricultural Research Council's Mass Spectrometer, to Mr B. W. E. Peaple for technical assistance and to Dr Helen Porter who made the radioactive starch from which the [^{14}C]glucose was prepared.

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Glutathione and Ascorbic Acid in the Metabolism of Germinating Peas. By S. P. Spragg and E. W. Yemm. (*Department of Botany and Biological Chemistry Laboratories, University of Bristol*)

Hopkins & Morgan (1943) have shown that reduced glutathione is rapidly formed in pea seeds during the early stages of germination. The activity of the tripeptide in enzymic systems of oxidation reduction (Mapson & Goddard, 1951), transpeptidation (Hanes, Hird & Isherwood, 1952) and glycolysis (Krimsky & Racker, 1952) indicates that it may play an important part in regulating cellular metabolism. This possibility has been explored by experiments in which the changes of glutathione, ascorbic acid and respiratory processes were studied in germinating pea seeds.

Evidence has been obtained that in the first stage of germination, 0–6 hr. in air at 22°, there is a rapid conversion of oxidized (GSSG) to reduced glutathione (GSH). The total amount of GSH + GSSG does not change significantly over the first 24–26 hr. of germination, suggesting that extensive synthesis or breakdown does not occur in these early stages. Ascorbic acid (AA) increases relative to dehydroascorbic acid (DHA) during the first 24 hr., but a loss of total AA + DHA is generally observed. Later both AA and DHA show substantial increases as germination proceeds.

The respiratory activities of the seed over the first 48 hr. of germination can be divided into three phases: (1) A rapid rise in O_2 uptake and CO_2 production during the uptake of water and extending over 6–9 hr. (2) A period from about 9 to 30 hr. in which respiration is maintained at a fairly steady rate. (3) A further increase of rate associated with the rupture of the testa of the seed. Several lines of evidence indicate that the interruption of the rising rate of respiration during phase (2) is in part attributable to the limitation of gaseous diffusion by the testa, and that at this stage a substantial part of the CO_2 arises from anaerobic glycolysis.

The increase in the proportion of the reduced forms of GSH and AA may be related to the restricted supply of oxygen to the tissues and the formation of GSH may play a significant part in the activation of —SH enzymes during the first phase of germination.

This work is part of the programme of the Food Investigation Organisation of the DSIR.

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Estimation of Amino Acids by Ninhydrin. By E. C. Cocking and E. W. Yemm. (*Department of Botany, University of Bristol*)

Moore & Stein (1948) and Smith & Agiza (1951) have described quantitative methods for estimating amino acids colorimetrically by means of the reaction with ninhydrin. Both these methods suffer from the disadvantage that equimolecular amounts of different amino acids do not produce equal colour intensities. More recently Troll & Cannan (1953) have modified the procedure so that a stoichiometric reaction occurs with quantitative yields of dioxohydrindylidene-dioxohydrindamine (DYDA), the probable end product of the reaction. For routine purposes the modified reaction has several disadvantages: it is difficult to control the pH accurately, high water blanks are obtained and it involves extensive use of a phenol–pyridine solvent. It has been possible to retain the advantage of the quantitative reaction of Troll & Cannan with a simple procedure suitable for routine analysis.

The method may be summarized as follows:

1 ml. of an amino acid solution containing 0·05–2·8 μg. amino nitrogen was mixed with 0·5 ml. citrate buffer pH 5 (0·2 M). The ninhydrin in methyl cellosolve (0·2 ml., 5 % w/v) and 0·01 M–KCN in methyl cellosolve (1 ml., 2 % v/v) were added to this solution either separately or as a single solution. Separately these reagents are stable for at least 1 month, and mixed, for at least 1 week. The well-mixed solution was heated for at least 15 min. at 100° and cooled for 5 min. in running tap water. The b.p. of the water–methyl cellosolve mixture is greater than 100°, and using tubes stoppered with a glass marble, evaporation losses during the heating period were negligible. The solution was made up to a convenient volume with ethanol (60 % v/v), and the optical density determined on a Unicam Spectrophotometer at 570 mμ, or the colour intensity read on an EEL colorimeter using filter 626. The colours were quite stable for at least 1 hr. at room temperature. It was found that most of the common amino acids gave colours equivalent to 100 ± 1 % of that of pure DYDA, except tryptophan (80 %) and lysine (110 %). Ammonia reacted yielding a colour equivalent to only 33 % of that of pure DYDA.

The following advantages are claimed for the above procedure:

(1) More complete reaction with amino acids gives greater sensitivity and more reproducible results.

(2) Lower sensitivity to ammonia results in small and consistent blank values.

(3) Shorter and less critical heating period is required.

(4) Considerable economy of ninhydrin: only about half the quantity is required compared with the method of Moore & Stein.

(5) It avoids the complication of storing reduced ninhydrin reagent under nitrogen.

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The Reaction of Disulphides with Sodium Sulphite. By R. Cecil and J. R. McPhee. (*Department of Biochemistry, Oxford*)

The reaction kinetics of four disulphide compounds with sodium sulphite have been studied, namely cystine, *NN'*-diacetylcystine diethyl ester, *NN'*-diformylcystine and dithiodipropionic acid. The equations are

$$RSSR + SO_3'' \rightleftharpoons RS^- + RSSO_3^-,$$
$$RS^- + H^+ \rightleftharpoons RSH,$$
$$SO_3'' + H^+ \rightleftharpoons HSO_3^-.$$

Reactions were carried out in buffered solutions at 25°. Samples were acidified with dilute nitric acid to pH≃2, and the thiol estimated by potentiometric titration with silver nitrate (Cecil & McPhee, to be published).

Acetylcystine ester shows the simplest behaviour because it has no ionizing groups. The reaction is found to be of the first order with respect to each reactant. It is reversible (Stricks & Kolthoff, 1951), the rate of the reverse reaction depending on the concentration of RS^-. At pH>9 this becomes significant, and the sulphite is all in the form SO_3''. By making use of the equilibrium constant under the same conditions, the specific rate constant for the forward reaction with SO_3'' can be calculated. This is found to be independent of pH.

At pH 4·5–7 the reaction is no longer reversible owing to the absence of RS^-, but the sulphite–bisulphite equilibrium has to be taken into account. The reaction mechanism is complex, and only the initial rates are considered. Apparent rate constants are found which decrease with decreasing pH. If both SO_3^{2-} and HSO_3^- react with disulphide then these apparent rate constants could be interpreted in terms of two specific rate constants, the pK_2 of sulphurous acid, and the pH. It was found that HSO_3^- does not react with these disulphides, and change in the apparent rate constants with pH can be accounted for by the corresponding change in SO_3^{2-} concentration. The specific rate constant agrees with that found in the higher pH range.

The other compounds show the same general behaviour, complicated only by the presence of charged groups. It is found that an overall negative change on the molecule greatly reduces the specific rate constant. Thus acetyl cystine ester gives $k=265$ l.mol.$^{-1}$ min.$^{-1}$, whereas diformyl cystine and dithiodipropionic acid give $k=9$ and $k=1{\cdot}1$ respectively. 'Neutral' cystine, i.e. in the zwitterion form, gives $k=1100$, whereas completely ionized cystine gives $k=3$. Thus at high pH cystine gives apparent rate constants which decrease with increasing pH. The specific rate constants for the different ionic species may be calculated from the apparent constants and the pK's of the amino groups.

One of us (J.R.M.) is indebted to the Commonwealth Government of Australia for a CSIRO traineeship.

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DEMONSTRATIONS

An Apparatus for Determining the Freezing-points of Small Volumes of Fluid. By G. R. Hervey (introduced by R. A. McCance). (*Medical Research Council Department of Experimental Medicine, University of Cambridge*)

The apparatus uses thermocouples and a potentiometer system to measure the freezing points of volumes of fluid of 0·1 ml. or less. The accuracy is 0·01° and the apparatus is reasonably simple to construct, and rapid and convenient to operate.

Paper Electrophoresis of Lipids and Lipoproteins of Human Sera. By W. G. Dangerfield and Elspeth B. Smith. (*Department of Pathology, St Bartholomew's Hospital, E.C.* 1)

The lipids on paper electrophoresis strips are stained with Sudan Black in 55 % (v/v) ethanol. This gives two main bands of staining, the α- and β-lipoproteins, each of which consists of two or more components. Further resolution of the bands can be achieved by extracting the unstained strips with organic solvents, which remove a large part of the lipid, particularly the neutral fat and cholesterol; this shows up other less soluble bands more clearly. A particularly characteristic insoluble pre-β-lipid band is found in serum from patients with type II nephritis.

Characteristic lipid patterns are found in a number of pathological conditions, and injection of heparin produces distinctive changes in the patterns.

The Liebermann reaction has been adapted for staining cholesterol on the paper; owing to the rapid uptake of moisture from the atmosphere the colour is not stable, but it lasts long enough to enable the strip to be photographed. Autoradiographs made from the sera of patients treated with ^{32}P show the possible distribution of phospholipid.

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

Normal human urinary porphyrins. By A. Comfort, H. Moore and M. Weatherall

Studies on sulphatases. 8. The arylsulphatase of a strain of *Alcaligenes metalcaligenes* isolated from inter-tidal mud. By K. S. Dodgson, T. H. Melville, B. Spencer and K. Williams

The reaction of metmyoglobin with strong oxidizing agents. By P. George and D. H. Irvine

Aerobic metabolism of the muscle of *Locusta migratoria*. By K. R. Rees

The titration curve of gelatin. By A. W. Kenchington and A. G. Ward

Oxidative phosphorylation in insect sarcosomes. By S. E. Lewis and E. C. Slater

Appendix—Relationship between amount of hexokinase and amount of phosphate esterified. By S. E. Lewis and E. C. Slater

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Incorporation of radioactive phosphate into lipids and pentosenucleic acid of cat brain slices. The effect of inorganic ions. By Muriel Findlay, W. L. Magee and R. J. Rossiter

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Actinomycete metabolism: origin of the guanidine groups in streptomycin. By G. D. Hunter, Mary Herbert and D. J. D. Hockenhull

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Changes in chemical composition during the development of 'cholesterol fatty livers'. By Jessie H. Ridout, C. C. Lucas, Jean M. Patterson and C. H. Best

Preventive and curative studies on the 'cholesterol fatty liver' of rats. By Jessie H. Ridout, C. C. Lucas, Jean M. Patterson and C. H. Best

Effects of lipotropic substances on the cholesterol content of the serum of rats. By Jessie H. Ridout, Jean M. Patterson, C. C. Lucas and C. H. Best

Iron metabolism in the laying hen. By W. N. M. Ramsay and E. A. Campbell

The biosynthesis and metabolism of betaines in plants:

2. The biosynthesis of glycinebetaine (betaine) in higher plants. By B. T. Cromwell and S. D. Rennie
3. Studies on the biosynthesis of precursors of glycinebetaine in seedlings of wheat (*Triticum vulgare* Vill.). By B. T. Cromwell and S. D. Rennie

The biosynthesis of proteins. 2. Synthesis of milk proteins by the goat. By Brigitte A. Askonas, P. N. Campbell and T. S. Work

Crystallization of goat β-lactoglobulin. By Brigitte A. Askonas

Addendum. Diffusion coefficient of crystalline goat β-lactoglobulin. By E. A. Caspary

Stimulation of respiration by 2:4-dinitrophenol. By E. C. Slater and S. E. Lewis

Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. By S. Hestrin and M. Schramm

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **331st Meeting** *of the Biochemical Society was held in the Department of Biochemistry, Queen's University, Belfast, on Friday,* 16 *July* 1954, *at* 11 *a.m. when the following papers were read:*

COMMUNICATIONS

The Structure of the Lysolecithin formed by the Action of Snake Venom Phospholipase *A* on Ovolecithin. By C. Long and I. F. Penny. (*Department of Biological Chemistry, University of Aberdeen*)

Previous work from this department has shown that the reactions of ovolecithin towards alkaline hydrolysis (Long & Maguire, 1953*a*) and on treatment with phospholipase *C* of *Clostridium welchii* Type *A* (Long & Maguire, 1953*b*) are consistent with the L-α configuration being assigned to it. Thus, it may be described as a diacyl L-α-glycerylphosphorylcholine, in which the two fatty acid radicals are esterified with the α′- and β-hydroxyl groups of the glycerol moiety. The enzyme phospholipase *A*, present in pancreas and in certain snake venoms, splits one fatty acid radical from lecithin to yield lysolecithin, but hitherto it has not been ascertained which of the two fatty acid radicals is removed. Splitting of the fatty acid radical from the α′-position will result in the exposure of a free primary alcoholic group, while if the fatty acid radical is split from the β-position a free secondary alcoholic group will be formed.

We have found that, in 2–5 hr. at room temperature, 0·1 N-$K_2Cr_2O_7$ in 10 N-H_2SO_4 will quantitatively oxidize primary alcohols to the corresponding carboxylic acids, four equivalents of $K_2Cr_2O_7$ being required. With secondary alcohols, the corresponding ketones are formed, with the uptake of two equivalents of $K_2Cr_2O_7$. Water-insoluble alcohols are first dissolved in redistilled chloroform, treated with the acid reagent and the mixture mechanically emulsified, the oxidation then proceeding to completion. The amount of $K_2Cr_2O_7$ used in the oxidation is determined by back-titration with standard ferrous ammonium sulphate, using diphenylamine as internal indicator.

Lysolecithin has been prepared from ovolecithin by the action of cobra-venom phospholipase *A* (kindly supplied by Dr J. W. Trevan, F.R.S., Wellcome Foundation Ltd.), following the method of Hanahan, Rodbell & Turner (1954). After catalytic hydrogenation, one mole reacted with four equivalents of $K_2Cr_2O_7$, indicating the presence of a primary alcoholic group. Thus, the fatty acid radical must have been split from the α′-position of ovolecithin by the action of this phospholipase *A*.

After this work was carried out, it was reported by Hanahan (1954) that lysolecithin is oxidized by $KMnO_4$ to a substance, which on acid hydrolysis yields a phosphoglyceric acid. This observation is also consistent with the structure of lysolecithin which we have proposed.

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The Mechanism of the Transformation of β-Carotene into Vitamin A *in vivo*. By J. Glover and E. R. Redfearn. (*Department of Biochemistry, University of Liverpool*)

Consideration of the formulae of the two molecules, β-carotene and vitamin A, has led many authors to regard the conversion reaction as a hydrolytic fission at the central double bond of β-carotene. Attempts to carry out this unusual reaction chemically have been unsuccessful and Hunter (1946) then suggested that probably oxidative attack at the central double bond is involved resulting in the formation of vitamin A aldehyde, which is later reduced to vitamin A alcohol.

β-Carotene is oxidized with hydrogen peroxide to vitamin A aldehyde (Hunter & Williams, 1945) which is obtained in good yield in the presence of a suitable catalyst (Wendler, Rosenblum & Tishler, 1950). We have studied this reaction in more detail and have observed that the initial attack takes place at a terminal double bond which is in keeping with physico-chemical theory (Zechmeister, Le Rosen, Schroeder, Polgár & Pauling, 1943). Since vitamin A aldehyde is reduced to

vitamin A alcohol *in vivo* (Glover, Goodwin & Morton, 1948), the idea of a reaction involving oxidation of the β-carotene followed by reduction of the vitamin A aldehyde produced, was strengthened. Still remaining to be determined, however, is the position of initial enzymatic attack. We have examined this problem.

Possible intermediates in the stepwise degradation of β-carotene from one end such as β-apo-8′-, β-apo-10′- and β-apo-12′-carotenals were prepared and administered to vitamin A deficient rats, and their metabolism studied. All were transformed into vitamin A, thereby confirming and extending the work of Euler, Karrer & Solmssen (1938). It was also noted that the β-apo-10′-carotenal undergoes β-oxidation to β-apo-12′-carotenol. The results indicate that the carotenals are oxidized by a type of β-oxidation to form vitamin A aldehyde. It has been shown that methyl branched fatty acids are oxidized *in vivo* when the methyl group is in the α- but not β-position to the carboxyl group (Kuhn & Livada, 1933; Carter, Osman, Levine & Gamm, 1939; Weitzel, 1951). The β-apo-8′- and -12′-carotenals above have methyl groups in the α-position, whereas vitamin A aldehyde is substituted in the β-position. Hence the latter is not oxidized from the open chain end but is readily reduced by alcohol dehydrogenase to vitamin A alcohol.

Again, hitherto unidentified substances related to the carotenoids (Festenstein, 1951), which have identical spectroscopic and chromatographic properties to the β-apo-10′- and -12′-carotenals, have been obtained from the horse intestine.

It would seem that provitamins A are first transformed into vitamin A aldehyde by stepwise oxidation from one end and then reduced to vitamin A. This mechanism is also favoured for reasons to be discussed. Experiments with ^{14}C-labelled β-carotene are in progress.

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The Synthesis of Methionine by Cell-free Extracts of *Bacterium coli*. By M. J. Cross and D. D. Woods. (*Department of Biochemistry, University of Oxford*)

Gibson & Woods (1952) have reported that cells of a mutant strain of *Bacterium coli*, requiring glycine or serine for growth, synthesize methionine when incubated in phosphate buffer, glucose, DL-homocysteine, DL-serine and cobalamin. Later, Gibson (1952) showed that the rate of synthesis is approximately halved if serine is replaced by glycine. With serine as one of the substrates, glycine was formed concurrently with methionine. This suggested that the overall reaction might consist of the reductive transfer of the β-carbon of serine to homocysteine yielding methionine. The β-carbon of serine is known to be a potential source of the methyl group of methionine in animals (Arnstein & Neuberger, 1953; Berg, 1953).

A cell-free extract which synthesizes methionine, apparently by such a reaction, has now been obtained from this mutant strain of *Bact. coli*. The most potent extracts were prepared by grinding the cells with alumina, but active extracts have also been obtained by shaking with glass beads and by ultrasonic disintegration. L-Methionine (20 mμmoles/hr./mg. protein; estimated by microbiological assay with *Leuconostoc mesenteroides* P.60) was formed when the extract was incubated in a mixture of DL-serine, DL-homocysteine, adenosine triphosphoric acid, hexose diphosphate (70% purity), pyridoxal phosphate, magnesium sulphate and phosphate buffer at 37°. The two amino acids were essential; the other substances increased the synthesis, but to varying extents.

Methionine synthesis was further increased (about 50%) by the addition of a heated extract of *Bact. coli*. This extract could not be replaced by a yeast extract prepared from Aluzyme according to Blakley (1954, and private communication).

A number of other compounds which take part in various one-carbon transfer reactions have been tested both in the presence and in the absence of heated *Bact. coli* extract. These included *p*-aminobenzoic acid, pteroylglutamic acid, tetrahydropteroylglutamic acid, leucovorin, diphosphopyridine nucleotide, and cobalamin; all were inactive.

In the presence of heated *Bact. coli* extract the rate of methionine synthesis was directly proportional to the concentration of enzymically

active extract. Glycine, also estimated by microbiological assay with *Ln. mesenteroides* P.60, was produced in approximately equimolar quantities to methionine, but only if homocysteine were present.

Formate neither replaced nor spared serine for the synthesis of methionine by the whole cells, and the incorporation of [^{14}C]formate into methionine in the presence of equimolar serine was very small (1 in 500 dilution). The cell-free enzyme system differed from that of Berg (1953) in the same way, i.e. formate will not replace serine. Glycine, whether formate was present or not, has 10 to 20% of the activity of serine.

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Active Transport of Water by Mitochondria. By C. A. Price* and R. E. Davies. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield*)

Mitochondria can maintain pre-existing water and ion gradients *in vitro* and accumulate certain ions (MacFarlane & Spencer, 1953; Stanbury & Mudge, 1953; Spector, 1953; Bartley & Davies, 1952, 1954). The fact that these activities are generally observed only under conditions favourable to oxidative phosphorylation suggested an investigation of the relations between water transport in mitochondria and the availability of 'high-energy' phosphate esters.

Analyses of rat liver mitochondria or pigeon breast muscle sarcosomes, separated rapidly (Bartley & Davies, 1954) from suspensions (up to 15 mg. dry wt./ml.), show that changes in the optical density of the suspensions at 520 mμ. (Cleland, 1952) and 950 mμ. with a 1 mm. light path correspond to changes in water rather than ion content (cf. Raaflaub, 1953*a*, *b*; Cleland, 1952). This modification of Cleland's method affords a rapid and essentially continuous estimation of mitochondrial water.

When rat liver mitochondria are shaken in air at 35° with 0·02 M succinate in the presence of 0·001 M adenosine triphosphate (ATP) or adenosine monophosphate (AMP), the rate of oxygen uptake is high (Q_{O_2} approx. -40 μl./mg. dry wt./hr.) and the particles retain their initially low water content (20% dry wt.). In the absence of adenosine phosphates the oxygen uptake is essentially unchanged in the first half-hour, but the mitochondria take up water (15% dry wt.). If swelling has not proceeded too far, the subsequent addition of 0·001 M AMP or ATP will cause the extrusion of water (from 17 to 20% dry wt.). This recovery is coincident with the resumption of oxidative phosphorylation. Ethylenediaminetetraacetate (3×10^{-3} M) also prevents swelling but does not cause extrusion of water.

Dinitrophenol (3×10^{-5} M) has only a slight effect on the maintenance of a low water content (MacFarlane & Spencer, 1953), but when added with 0·001 M ATP to swollen mitochondria prevents the active extrusion of water. At higher concentrations (10^{-4} M) dinitrophenol causes rapid swelling of the particles in the presence or absence of ATP.

These results indicate that active transport of water by mitochondria requires 'high-energy' phosphate esters.

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Studies on the Absorption and Metabolism of Ergosterol in the Guinea Pig. By J. Glover, W. M. F. Leat and R. A. Morton. (*Department of Biochemistry, University of Liverpool*)

Guinea pig intestine possesses an enzyme which maintains provitamin D_3 in equilibrium with the tissue cholesterol (Glover, Glover & Morton, 1952). It is of interest to determine (*a*) if this cholesterol dehydrogenase can also hydrogenate ergosterol or (*b*) if the enzyme will be inhibited by it. Answers to these problems could have a bearing on the selective action of the intestine in excluding dietary phytosterols (Schönheimer, 1931) each of which has a side chain different from that of cholesterol.

Ergosterol (dose, 150 mg.) in ethyl oleate, was administered to groups of young guinea pigs. After various time intervals, the animals were killed and the concentration of cholesterol and of 7-dehydrosterol in the small intestines and livers determined. No significant difference was observed in the total sterol contents of the organs of the dosed animals, from those of controls even 24 hr. after dosing but the quantity of 7-dehydrosterol in the livers rose above the control level during absorption to a value representing approximately 0·04–0·13% of the dose. No change in the 7-dehydrosterol content of the intestines could be detected.

The experiments were repeated using uniformly labelled [^{14}C]ergosterol (dose 3–200 mg. $\equiv$ 0·3–1·4 μc) (Glover & Leat), dispersed in ethyl oleate or in aqueous (Tween 80) solution. Groups of animals were sacrificed at 6 and 17 hr. after dosing. The results showed that only small amounts of the administered material had been absorbed. In terms of ^{14}C-radioactivity, the unsaponifiable matter from the livers, intestines and adrenals of the various groups contained 0·13 to 0·72%; 0·28 to 2·6%; and 0·03 to 0·32% respectively of the original dose. The fatty acid fractions from the above tissues contained respectively from a trace up to 0·76%; 0·05–1·22% and 0·04–0·2% of the dose. Other tissue lipids were also examined for radioactivity but no appreciable amounts were detected. These results are in general agreement with the findings of Hanahan & Al-Wakil (1953) on the absorption of ergosterol in the rat.

The radioactivity in the intestinal unsaponifiable matter was mainly due to ergosterol. Evidence for the reduction of ergosterol to 7:8-dihydroergosterol (brassicasterol) has not been obtained. The radioactive fatty acid fractions have not been fully investigated, but similar results from *in vitro* studies suggest that the metabolite(s) of ergosterol present is formed by the tissue enzymes and not by intestinal bacteria.

In conclusion, the results show that the small amount of ergosterol absorbed (<4% of the dose) is not hydrogenated in the small intestine as is 7-dehydrocholesterol, but is metabolized (oxidatively?) quite quickly (about 50% in 17 hr.). Furthermore, the ergosterol does not appear to disturb the cholesterol/7-dehydrocholesterol equilibrium.

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The Distribution of Cholesterol and 7-Dehydrocholesterol in the Intestinal Mucosae of the Guinea Pig. By J. Glover and C. Green. (*Department of Biochemistry, University of Liverpool*)

In a further study of the cholesterol/7-dehydrocholesterol equilibrium in the intestinal mucosae of the guinea pig (Glover, Glover & Morton, 1952), the distribution of these sterols in the tissue cells was examined. The mucosal layers of the small intestine were separated from the muscle layers as previously described (*loc. cit.*) except that the whole tissue was maintained at 5° approx. The mucosal preparation was suspended in 5–7 volumes of 0·25 M sucrose and comminuted in a Potter-Elvehjem pestle homogenizer. The cell components of different particle size were fractionated at 3 to 7° in a high speed refrigerated centrifuge by a modification of the method described by Schneider & Hogeboom (1950). The various fractions designated 'cell debris + nuclei', 'mitochondria', 'microsomes' and 'ground-plasm' were obtained and the quantities of cholesterol and 7-dehydrocholesterol determined in each fraction. The 'microsomes' contained both sterols in the greatest amount. In the fasting animals, the proportion of cholesterol to 7-dehydrocholesterol was found to be 9 to 12/1 in each of the fractions. Then groups of 2 young guinea pigs in the same weight range (250–350 g.) were dosed with pure 7-dehydrocholesterol (150 mg./animal) dispersed in ethyl oleate. Five hours after dosing, the distribution pattern of the two sterols in the mucosal tissue was again determined as above. The ratios of the two sterols in the various fractions were found to agree quite closely as before but were lower (6 to 10/1) than those for the fasting animals due to the presence of the absorbed 7-dehydrocholesterol. These results imply that the endogenous sterols can exchange rapidly with the 7-dehydrocholesterol undergoing absorption in the small intestine, which incidentally has a more active sterol turnover (Popják & Beeckmans, 1950) than any other organ.

This exchange may be important in connexion with the absorption of animal sterols and the extremely low absorption of phytosterols, which at the same time can reduce or block cholesterol absorption. A phytosterol with additional branching in the side chain compared to cholesterol may not fit easily, say with the other components of the cell membrane or with another protein carrier and hence tend to inhibit the cholesterol exchange which may be necessary for its absorption. This

hypothesis has been confirmed with ^{14}C-labelled sterols.

Esterified, cholesterol or 7-dehydrocholesterol was not detected in any cell fraction. However, traces were detected in the ground-plasm fraction from animals which had been on a cholesterol diet for several days. This is in agreement with previous work in this laboratory and with the results of Favarger & Metzger (1952).

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The Use of Homocysteine in the Estimation of Dehydroascorbic Acid. By R. E. Hughes. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

It has been found that at pH values above 6·5, dehydroascorbic acid is rapidly reduced by homocysteine to ascorbic acid, and that under certain simple conditions the ascorbic acid so formed can be estimated with 2:6-dichlorophenol-indophenol dye, without any interference from the excess of reductant (homocysteine). These findings form the basis of a method developed for the estimation of dehydroascorbic acid.

The material under examination is extracted with 3 % (w/v) metaphosphoric acid, and an estimate of the ascorbic acid content of the extract is made by measuring photoelectrically the reduction of 3 ml. of 0·01 % (w/v) indophenol dye (1 ml. ≡ 0·01 mg. ascorbic acid) by 3 ml. of the extract in the presence of 4 ml. of phosphate–citrate buffer of pH 2·5 (Bessey, 1938). With solutions containing high concentrations of ascorbic acid, a dye of strength 0·02 % may be used. A second 3 ml. sample of the extract is treated with 1 ml. of homocysteine solution (about 1·5 mg./ml.), and the quantity of 45 % (w/v) K_2HPO_4 required to change the pH to 7·0 slowly added from a microburette. The reduction of the dehydroascorbic acid is complete after 12 min. at room temperature, and the reaction mixture is then acidified by the addition of 2 ml. of 30 % (w/v) metaphosphoric acid. The total ascorbic acid is determined photoelectrically as described above, and the dehydroascorbic acid content obtained by subtraction of the original ascorbic acid value. Provided that the indophenol dye reduction is carried out at pH 2·5, and that the galvanometer is read within 20–30 sec. of the addition of the dye, there is no interference from the homocysteine present.

Recoveries of 95–98 % are obtained from solutions of chemically prepared dehydroascorbic acid and from mixtures with ascorbic acid.

The method is not completely specific for dehydroascorbic acid, but the specificity can be extended by adding boric acid (0·5 %) to the reaction mixture. At pH 7·0 there is no reduction of dehydroascorbic acid by homocysteine after the addition of boric acid, because of the powerful catalytic effect of the borate ion on the breakdown of dehydroascorbic acid to the difficultly reducible dioxogulonic acid (Huelin, 1949). Thus, dehydroascorbic acid can be distinguished from certain interfering compounds (e.g. dehydroreductic acid), the reduction of which by homocysteine is not appreciably affected by the presence of boric acid.

Examples are given of the application of this technique to various plant and animal tissues including the plasmas of different species.

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Cholinesterase Inhibition by Lysergic Acid Diethylamide. By R. H. S. Thompson, A. Tickner and G. R. Webster. (*Department of Chemical Pathology, Guy's Hospital Medical School, London*)

Little is at present known of biochemical changes that may accompany the psychological effects produced by administration of lysergic acid diethylamide to man. The action of this compound on a number of mammalian esterases has therefore been studied. It has been shown that concentrations of the order of 10^{-6} M cause 50 % inhibition of human plasma cholinesterase. Tributyrin hydrolysis by human plasma is inhibited to about the same degree. On the other hand the true cholinesterase in human erythrocytes is not significantly affected by 10 times the concentration required to produce 90 % inhibition of the plasma enzyme.

It has been shown that this inhibition of the plasma cholinesterase is competitive, and is readily reversible.

Pseudo-cholinesterase activity in a number of different areas of the human brain is also inhibited (approx. 60 % by 5×10^{-6} M). As in the erythrocytes, true cholinesterase in the brain is unaffected by these concentrations. Tributyrin hydrolysis by

brain is also largely unaffected, confirming the earlier observations of Ord & Thompson (1952) that pseudo-cholinesterase in brain, in contrast to that in the plasma, does not contribute to any significant degree to the hydrolysis of tributyrin by this tissue.

The pseudo-cholinesterase of human brain appears to be more sensitive to inhibition by LSD than the corresponding enzyme in the brains of the other animal species which we have studied. Thus, butyrylcholine hydrolysis by rat, guinea pig and chicken brain is hardly affected by concentrations which produce 80–90 % inhibition in human brain. Rabbit and monkey brain pseudo-cholinesterase are slightly more sensitive, though in each case considerably less so than the corresponding enzyme in human brain.

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The Effect of Cholinesterase Inhibitors on The Leakage of Potassium from Brain Slices. By K. P. Strickland* and R. H. S. Thompson. (*Department of Chemical Pathology, Guy's Hospital Medical School, London*)

Rothenberg (1950) has reported that the cholinesterase inhibitors di*iso*propyl phosphofluoridate (DFP) and eserine increase the overall permeability of squid giant axons toward sodium and potassium. Lindvig, Greig & Peterson (1951) have further indicated that the erythrocyte requires, among other factors, an adequate acetylcholine–cholinesterase system to prevent a gain of sodium or a loss of potassium. Because of this possible relationship between the cholinesterase system and the ion permeability of the nerve cell membrane, the effects of cholinesterase inhibitors (DFP, eserine and the prostigmine analogue) on the leakage of potassium from brain slices have been investigated. Slices of chicken brain were incubated in a bicarbonate–glucose–glutamate medium at pH 7·4 under aerobic conditions (Terner, Eggleston & Krebs, 1950). Following incubation, the potassium of the suspending medium was measured by flame photometry. DFP, eserine and Nu 1250 at concentrations of 2×10^{-2}M, 2×10^{-2}M and 2×10^{-4}M respectively were found to increase the outflow of potassium from the slices. Concentration ten times less than these caused no significant increase in the loss of potassium from the slices.

Minimum concentrations of these inhibitors which cause complete inhibition of cholinesterase activity of chicken brain slices were found to be (*a*) DFP 10^{-5}M, eserine 10^{-4}M and Nu 1250 10^{-5}M, when acetylcholine perchlorate was used as substrate to measure the total cholinesterase activity, (*b*) DFP 10^{-5}M, eserine 10^{-5}M and Nu 1250 10^{-6}M with β-acetyl-methylcholine chloride as substrate for the true cholinesterase, and (*c*) DFP 10^{-6}M, eserine 10^{-5}M, and Nu 1250 10^{-5}M with butyrylcholine perchlorate as substrate for the pseudo-cholinesterase.

The concentrations of inhibitors required to produce complete inhibition of the true cholinesterase are in every case very much less than the minimum concentrations required to cause potassium leakage. With DFP and eserine the same relationship also holds for the pseudo-cholinesterase. This clearly suggests that for chicken brain slices under our conditions there is no correlation between complete cholinesterase inhibition and the first observed loss of potassium from the slices poisoned with either DFP, eserine or Nu 1250.

Assuming that an increased potassium loss indicates an increased permeability these results seem to dissociate the dependence of the permeability of the nerve cell membrane on cholinesterase activity rather than lend support to Rothenberg's suggestion or to the conclusion of Lindvig, Greig & Peterson (1951) that permeability is dependent on cholinesterase activity.

* Post-doctoral Fellow, National Research Council of Canada.

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The Enzymic Formation of Glyoxylate and Succinate from Tricarboxylic Acids. By H. J. Saz.* (*A.R.C. Unit of Microbiology, Department of Microbiology, Sheffield University*)

In a recent note, Campbell, Smith & Eagles (1953) reported the presence of an enzyme system in sonic extracts of *Pseudomonas aeruginosa* which catalysed the anaerobic dissimilation of either citrate or *cis*-aconitate into glyoxylate and succinate. *Iso*citrate was not attacked by their preparation.

Extracts of both *Ps. aeruginosa* and *Ps. fluorescens* prepared with the Mickle disintegrator

contain this enzyme system. In contrast to the preparations of Campbell, Smith & Eagles (1953) these preparations attacked (±)-*iso*citrate as well as citrate and *cis*-aconitate. The identity of the glyoxylate was confirmed by the isolation of the 2:4-dinitrophenylhydrazone, and determination of the melting point. Succinate and glyoxylate were formed in equivalent amounts.

Heated preparations as well as ammonium sulphate fractions were obtained which were two to five times as active on *cis*-aconitate as on either (±)-*iso*citrate or on citrate. But, when (+)-*iso*citrate, the naturally occurring isomer, was used in place of synthetic (±)-*iso*citrate the activity was greater on this substrate than on *cis*-aconitate, suggesting that (+)-*iso*citrate is the true substrate of this enzyme system.

Crude extracts prepared by crushing cells in the Hughes press (Hughes, 1951) formed citrate from succinate and glyoxylate, showing that the reaction is reversible. These preparations contained aconitase.

Glyoxylate formation is strongly inhibited by pyrophosphate, ethylenediaminetetraacetic acid (Versene), and high concentrations of cyanide. Boiled extracts of dried brewer's yeast stimulated glyoxylate production. Dialysis or ashing of the yeast extract resulted in complete loss of stimulating activity. No glyoxylate was formed on incubation of the yeast extract with the enzyme. These results suggest a requirement for both an organic and an inorganic cofactor.

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The Biosynthesis of a New Vitamin B_{12} Analogue. By K. H. Fantes and Cynthia H. O'Callaghan. (*Glaxo Laboratories Ltd., Sefton Park, Stoke Poges, Bucks*)

Dulaney & Williams (1953), by adding *o*-phenylenediamine (OPD) or any one of a number of related substances to a synthetic medium, claimed to have doubled or trebled vitamin B_{12} production by *Streptomyces griseus* and some other species, as judged by the *Escherichia coli* (mutant) plate assay. Using OPD labelled with deuterium these authors showed that not more than 20% of the isolated vitamin was labelled and from this concluded that the titre increases were not due to the compound acting as a B_{12} precursor. It was shown by the authors that growth of the organism was initially inhibited by OPD. In order to overcome this inhibition, it is postulated, the organism produces an antagonist to OPD, namely vitamin B_{12}.

We have re-investigated this problem and have found by chromatographic and bio-autographic techniques that the addition of OPD to the medium caused the production of a new substance active for *Esch. coli* and *Lactobacillus leichmannii* 313. Increasing levels of OPD gave rise to increasing levels of the new growth factor at the cost of vitamin B_{12} production. The biosynthesis of the new factor in the presence of OPD was prevented by the simultaneous addition to the medium of relatively low levels of 1:2-diamino-4:5-dimethylbenzene, a postulated vitamin B_{12} precursor (Woolley, 1950, 1951).

The new growth factor was isolated as red needles. Its absorption spectrum shows the typical vitamin B_{12} peak at 361 mμ. and is in general closely similar to that of vitamin B_{12}. For equal optical absorptions at 361 mμ. the new substance is 2·4 times as active as vitamin B_{12} in the *Esch. coli* plate assay and 1·8 times as active in the *Lb. leichmannii* plate assay.

Benziminazole was identified as one of the new material's acid hydrolytic products by its R_F and its absorption spectrum: it is almost certainly identical with the one just obtained independently by a quite different approach (Ford, Holdsworth & Kon, 1954). These authors incubated *Esch. coli* and factor B in the presence of benziminazole and by chromatography demonstrated the formation of a new compound with an R_F value just less than that of vitamin B_{12}.

Clinical trials kindly carried out by Professor L. J. Witts and Dr Sheila Callender at the Radcliffe Infirmary, Oxford, showed that the new material was fully active in three patients with pernicious anaemia. The lowest level so far tried was a single dose of 10μg.; the new material therefore appears to be not less active than vitamin B_{12} itself.

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Initiation of Endogenous Fermentation in Baker's Yeast by Azide in High Concentration. By T. G. BRADY and P. F. DUGGAN. (*Department of Biochemistry, University College, Cork*)

It has generally been found that a yeast suspension in the absence of added glucose is unable to ferment its carbohydrate reserves (cf. Stier & Stannard, 1936; Spiegelman & Nozawa, 1945), and that sodium azide, added usually in concentrations ranging from 10^{-3} to 10^{-5} M, leads neither to utilization of reserve carbohydrate, nor to CO_2 nor ethanol formation (cf. Trevelyan, Gammon, Wiggins & Harrison, 1952; Fales, 1953). Rothstein & Berke (1952) have, however, reported that CO_2 and ethanol were formed in small amount from reserve carbohydrate in the presence of 10^{-3} to 10^{-5} M sodium azide (the largest amounts being produced at the higher concentration) but considerably greater quantities were produced in the presence of 2:4-dinitrophenol in equivalent concentration.

In a study of the effect of inhibitors on phosphorus distribution in yeast during the fermentation of glucose, inhibitor concentrations were employed giving approximately 50% reduction in the fermentation rate. When sodium azide in such concentration (2×10^{-2} to 10^{-2} M) was employed it was found that the control containing sodium azide but no glucose gave an increased CO_2 output compared to the control with no azide.

Further investigation of this effect has shown it to be a fermentative type of reaction. Ethanol, in considerable quantity, is formed and there is also a decrease in stored carbohydrate.

The endogenous fermentation of yeast suspensions in the presence of sodium azide in concentrations ranging from 2×10^{-1} to $1{\cdot}25 \times 10^{-3}$ M has been measured and a maximum liberation of CO_2 and formation of ethanol was obtained at a concentration of approximately 10^{-2} M sodium azide. The fermentation was also shown to be optimal between pH 4·5 and 5·0, falling steeply on either side. At pH 3·5 there was a 50%, and at pH 5·5, a 25% decrease in ethanol formation. The fact that the reaction sequence is pH-dependent is taken as evidence that, at least, one of the participating enzymes is located in the outer metabolic region or membrane of the yeast cell because H ions cannot penetrate further than this region (cf. Conway & Downey, 1950).

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Comparative Studies of Some Sugar-Phosphate fractions of Early Post-natal and Normal Milks. By F. E. HYTTEN, F. G. REITHEL, E. FLETCHER and F. H. MALPRESS. (*Departments of Midwifery, Foresterhill, University of Aberdeen; Chemistry, University of Oregon, U.S.A. and Biochemistry, Queen's University, Belfast*)

McGeown & Malpress (1952) have reported the occurrence of galactose 1-phosphate, glucose 1-phosphate, glucose 6-phosphate and a lactose phosphate in trace amounts in barium–ethanol precipitates from deproteinized cow's milk. Similar studies have now been carried out on comparable fractions from goat and human milks, and from cow, goat and human post-natal milks collected in the first 24 hours after parturition. All the samples from ruminants showed basically the same ester pattern, though quantitatively the ester concentrations were considerably increased in the 'colostrum' samples. All the esters found, except glucose 6-phosphate, were acid-labile and chromatographic analysis of the sugar moieties after the action of either alkaline phosphatase or mild acid hydrolysis gave almost identical results; this fact suggests that these samples were not contaminated to any great extent with non-phosphate, acid-labile sugar compounds.

By contrast, both the post-natal and the normal human milks gave preparations which, while showing the presence of galactose 1-phosphate and glucose 6-phosphate, gave no sure indication of the other two esters found in ruminant milks, while mild acid hydrolysis, but not the action of phosphatase, liberated relatively large amounts of fucose, together with more galactose; there was no increase in free phosphate which could be related to the appearance of these sugars.

The forms in which fucose occurs in milk have yet to be established, but the presence of fucose-containing oligosaccharides in human milk has already been reported by Gyorgy and his co-workers (see

Gauhe, Gyorgy, Hoover, Kuhn, Rose, Ruelius & Zilliken, 1954), who comment on their function as specific growth factors for a variant of *Lactobacillus bifidus*. The structural relationship of fucose to galactose suggests that these compounds might also be involved in lactose synthesis.

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Liver Composition after Growth Hormone Treatment or Adrenalectomy. By E. Reid. (*Chester Beatty Research Institute, Royal Cancer Hospital, London, S.W.* 3)

Analyses have now been performed on the livers of female rats, fasted 16 hr., and previously subjected to adrenalectomy (1 % NaCl in drinking water) or to treatment for 2 weeks with pituitary growth hormone and kept on a constant food intake. Following perfusion of the liver *in situ* with 0·25 M sucrose solution, a minced sample was homogenized in this medium and subjected to differential centrifugation. The 'nuclear' fraction (containing some unbroken cells), and a portion of the cytoplasmic fraction, were precipitated with trichloroacetic acid, defatted and dried; the residues were analysed for ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) using the Schmidt-Thannhauser procedure. The remainder of the cytoplasmic fraction was further centrifuged to give 3 sub-fractions (mitochondria, microsomes and supernatant), each of which was dialysed and freeze-dried.

The experimental rats were compared with untreated control rats with respect to the yields (per 100 g. body wt.) of the 'nuclear' and cytoplasmic residues and of the sub-fractions, the concentrations of RNAP in the 'nuclear' and cytoplasmic residues and of DNAP in the 'nuclear' residue, and the overall RNAP/DNAP ratio. The yield of the 'nuclear' residue was increased after growth hormone treatment, and the yield of the mitochondrial sub-fraction was decreased after adrenalectomy. No other significant changes have so far been observed. Both these changes were observed when growth hormone was given to adrenalectomized rats, and there was a rise in the liver-wt./body-wt. ratio; the increase in body wt. was comparable with that obtained in intact rats given growth hormone.

In the case of kidney, there was no increase in the yield of the 'nuclear' residue after growth-hormone treatment or decrease in the yield of the mitochondrial sub-fraction after adrenalectomy.

I thank the British Empire Cancer Campaign for awarding a Fellowship, and Dr S. L. Steelman (of Armour & Co., Chicago) for providing the growth hormone.

Ribonucleic Acid Phosphorus and Deoxyribonucleic Acid Phosphorus in Hypertrophied Kidney Tissue. By A. B. Morrison and W. M. Pearce. (*Department of Experimental Medicine, Medical Research Council, University of Cambridge*)

The right kidney and two-thirds of the left kidney were removed from 16 male rats all weighing about 250 g., using the technique described by Platt, Roscoe & Smith (1952). The animals were divided into four groups of four and three similar sized groups of animals served as sham operated controls. Two months after the operation the animals were killed and the remaining renal fragment was removed from each of the experimental animals and the cortex dissected free from the medulla. The cortical tissue from all four animals in a group was pooled and analysed for ribonucleic acid phosphorus (RNA.P) and deoxyribonucleic acid phosphorus (DNA.P) by the method of Schmidt & Thannhauser (1945). At the same time the left kidneys were removed from the control groups and treated similarly.

There was an increase in DNA.P in the experimental groups when expressed per 100 g. of tissue and compared with the corresponding fractions of the control group. The increase in DNA.P was proportionally greater than the rise in RNA.P. This is shown clearly by the RNA.P/DNA.P ratio, which averaged 1·9 in the experimental group and 2·5 in the controls. The results suggest an increase in the number of cells indicating a true hyperplasia.

Mandel, Mandel & Jacob (1950) examined the kidney remaining after unilateral nephrectomy in rats. They analysed the renal tissue thirty days after the operation and found that the increase in RNA.P was greater than that in DNA.P. They interpreted their findings as being due to an increase in cell size.

Subtotal (5/6) nephrectomy may cause greater hyperplasia in the remaining renal tissue than

unilateral nephrectomy by increasing the amount of metabolic end products to be excreted per unit of functioning renal tissue. However the analyses described here were carried out on tissue which had been undergoing hypertrophy for two months instead of one month as in the animals examined by Mandel & Jacob and in compensatory growth of the kidney the cells may well first increase in size and later proliferate.

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Biosynthesis of Vitamin B_{12}-like Compounds. By J. E. Ford, E. S. Holdsworth and S. K. Kon. (*National Institute for Research in Dairying, University of Reading*)

In further work on the biosynthesis of B_{12} vitamins (Ford & Holdsworth, 1954), *Bacterium coli* mutant 113–3 was given Factor B (see Gant, Smith & Parker, 1954) with a variety of different compounds structurally related to benziminazole or adenine. Of several new B_{12} vitamins formed, that produced with benziminazole has been isolated in pure form. It is active microbiologically for *Bact. coli* and *Lactobacillus leichmannii*, and also for *Ochromonas malhamensis*, a protozoan that has hitherto been regarded as highly specific in its requirement for cyanocobalamin (Ford, 1953). The compound is almost certainly identical with that isolated by Fantes & O'Callaghan (1954) from cultures of *Streptomyces griseus* grown with *o*-phenylenediamine.

With 5:6-dichlorobenziminazole a new B_{12} vitamin was formed, having the same order of activity as cyanocobalamin for the three micro-organisms.

These two compounds are intrinsically active and not by virtue of conversion to other known forms of 'B_{12}'.

Other new B_{12} vitamins were formed with 5-methylbenziminazole*, 5-aminobenziminazole, 5-nitrobenziminazole*, 4-chloro-1:2 benztriazole*, benzthiazole*, 2:8-dichloroadenine* and 2:6-diaminopurine. All were active for *Bact. coli*, and those marked with an asterisk also, in varying degree, for *Ochromonas*.

It seems likely that the 'B_{12}' requirement of *Bact. coli* mutant 113–3 is imposed by a defect in the synthesis of Factor B. Given this factor, the mutant seems to behave in the same fashion as certain 'wild' species of coliform organisms.

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A Nitrogenous Derivative of Lactose from Lactating Rat Mammary Gland. By R. Heyworth and J. S. D. Bacon. (*Department of Biochemistry, The University, Sheffield*)

Rat mammary gland contains an oligosaccharide (cf. Caputto & Trucco, 1952) with R_F 0·03 in the butanol–acetic acid–water solvent of Partridge (1948), and 0·16 in the *iso*butyric acid–ammonia solvent of Krebs & Hems (1953). This substance, which is most abundant in early lactation, was not detected in guinea pig glands.

Glands at 4–11 days lactation were chopped into boiling water, the extract deproteinized with 0·5 vol. ethanol and 0·25 vol. chloroform, and the substance separated from lactose by gradient elution with aqueous ethanol from charcoal–Celite columns (Bacon & Bell, 1953), yielding preparations with about 150 mg. dry wt./100 g. fresh tissue.

The preparations contained nitrogen (1·2–3·7 %) but no phosphate, sulphur or halogen; the absorption spectrum showed no peaks (215–440 mμ.).

Reducing sugar estimation (Nelson, 1944; Somogyi, 1945); the silver nitrate spray (Trevelyan, Procter & Harrison, 1950) and the benzidine–acetic acid spray (Horrocks & Manning, 1949) suggested a free reducing group. This was confirmed by the formation of an *N*-benzylglycosylamine under the conditions of Bayly & Bourne (1953). When run in butanol–acetic acid after treatment with benzylamine the compound appeared unchanged. Paper chromatography indicated that acid hydro-

lysis (0·5N-H_2SO_4, 80°) yields, after 10 min., lactose, and later, glucose and galactose. The reducing sugar content of six preparations, calculated as lactose, ranged from 23–36 %.

The substance moved to the anode under paper electrophoresis at pH 7·0 and 2·2. Titration curves suggested groups with pK below 2·5 and above 12.

Following the report of Trucco & Caputto (1954), the preparations were found to give purple colours on heating with Bial's reagent (0·25 % (w/v) orcin, 0·01 % (w/v) $FeCl_3$ in 12N-HCl) and with Ehrlich's reagent (3 % (w/v) *p*-dimethylaminobenzaldehyde in 6N-HCl). Acid hydrolysis (0·5N-H_2SO_4, 80°, 10 min.) gave a strong Ehrlich-positive spot with R_F values, 0·56 in the ethyl acetate–pyridine–water solvent of Trucco & Caputto, (1954); 0·25 in butanol–acetic acid; and 0·42 in phenol.

Alkaline hydrolysis (0·25N-NaOH, 80°, 30 min.) gave a number of Ehrlich-positive spots. Two of these (R_F 0·39, 0·85; butanol–acetic acid) absorbed u.v. light; one (R_F 0·85), present in traces, was provisionally identified as 2-carboxypyrrole (cf. Hiyama, 1949).

Neither glucosamine nor *N*-acetylglucosamine was detected in hydrolysates.

The compound is probably the 'neuraminlactose' of Trucco & Caputto (1954).

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The Phosphorus-containing Components of Some *Mycobacteria*. By F. G. Winder and Joan Denneny. (*Medical Research Council of Ireland Laboratories, Trinity College, Dublin*)

The contents of acid-soluble phosphorus, lipid phosphorus, pentosenucleic acid, deoxypentosenucleic acid, metaphosphate, and phosphoprotein, in *Mycobacterium smegmatis*, *M. phlei*, and *M. tuberculosis* R 1 Ra and H 37 Ra, have been determined. The results of these determinations will be presented.

Altered Phosphorus Metabolism of Some *Mycobacteria* in the Presence of Certain Agents. By F. G. Winder and Joan Denneny. (*Medical Research Council of Ireland Laboratories, Trinity College, Dublin*)

In the presence of certain agents (for example, 2 % tetrahydrofurfuryl alcohol) the phosphorus metabolism of the *Mycobacteria* studied was considerably altered. The most striking effects observed were a rapid formation and accumulation of meta phosphate. This accumulation continued until a level several times higher than normal was reached. The results of some studies on this altered phosphorus metabolism will be presented.

The Preparation of Heparinase. By L. B. Jaques and M. H. Cho. (*Department of Physiology, University of Saskatchewan, Saskatoon, Sask.*)

An enzyme which destroys the anticoagulant activity of heparin was extracted from rabbit liver by Jaques in 1940. Jaques & E. Keeri-Szanto (1952) have described a simple method for following the activity of the enzyme based on the method developed by Monkhouse & Jaques (1950) for the determination of heparin in blood. Studies with this method have been conducted on the preparation of active enzyme extracts. The enzyme could be extracted from tissue either with glycerol, saline or water. On precipitation with ammonium sulphate, the most active fraction was precipitated at 30–50 % saturation and pH 7·0. With ethanol, the optimal conditions were pH 5·5, NaCl concentration 0·1–0·2 % and 7 % ethanol; and for acetone, pH 8, NaCl 0·1 %, acetone 70 %. Enzyme activity was

precipitated in two fractions (corresponding to the lowest and highest concentrations of reagent) on addition of increasing amounts of ammonium sulphate, acetone, and alcohol.

Active extracts were obtained from livers of ox, human, rat and gopher (*Citellus richardsonii*), and from ox, pig and rabbit kidney, but not from dog liver. On testing for localization of enzyme in the cell components of rat and rabbit liver by differential centrifugation, the activity was found mainly associated with submicroscopic particles and supernatants, although nuclei and mitochondrial fractions showed some enzyme activity.

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DEMONSTRATIONS

Techniques for the Maintenance and Biochemical Analysis of Cultures of Human and Avian Cells. By I. LESLIE and W. C. FULTON. (*Department of Biochemistry, The Queen's University, Belfast*)

Paper Chromatography of Phosphate Esters By E. FLETCHER and F. H. MALPRESS. (*Department of Biochemistry, Queen's University, Belfast*)

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **332nd Meeting** *of the Biochemical Society was held in the Department of Biochemistry, The University, Sheffield* 10, *on Friday, September* 24*th*, 1954, *at* 11 *a.m. when the following papers were read:*

COMMUNICATIONS

SH Groups in Haemoglobins. By V. M. Ingram. (*Medical Research Council Unit for the Study of the Molecular Structure of Biological Systems, Cavendish Laboratory, Cambridge*)

Recent X-ray investigations by Green, Ingram & Perutz (1954) of Ag and Hg mercaptide derivatives of horse haemoglobin made it desirable to redetermine the number of available SH groups in this and related haemoglobins. For this purpose amperometric titration methods using $AgNO_3$ (Benesch & Benesch, 1948; Ingbar & Kass, 1951) and $HgCl_2$ (Kolthoff, Stricks & Morren, 1954) were employed.

Each mole of native horse oxyhaemoglobin was found to bind either 4 Ag or 2 Hg atoms. When the protein was first combined with 1 mole of *p*-chloromercuribenzoate (PCMB), titration with $AgNO_3$ showed only 2 SH groups to be left; combination with 2 moles of PCMB removed all SH groups detectable by $AgNO_3$. The same holds true when $HgCl_2$ is used instead of PCMB. Native ox and human oxyhaemoglobins behaved in a similar manner, whilst native sheep oxyhaemoglobin took up 8 Ag atoms or 4 Hg atoms per mole of protein. It was interesting to find that when ox, human or sheep haemoglobins were first combined with a mercurial, each Hg atom would block 2 SH groups to $AgNO_3$, no matter whether $HgCl_2$, PCMB, or CH_3HgNO_3 were used.

In horse haemoglobin denatured with sodium dodecyl sulphate (SDS, molar ratio 200:1) 6 SH groups per mole could be detected by $AgNO_3$ titration, which is the maximum number possible judging by the total sulphur and the methionine contents (Tristram, 1953). The denatured protein bound 4 $HgCl_2$. Blocking before denaturation with 1 $HgCl_2$ per mole reduced the number of SH groups found by $AgNO_3$ to approximately 3, but when 2 $HgCl_2$ were used there was hardly any uptake of Ag. Nevertheless horse haemoglobin blocked with 2 $HgCl_2$ still took up another 2 $HgCl_2$ when titrated in the presence of SDS. If the protein is first combined with increasing proportions of PCMB up to 4 molecules, and then denatured, it was found that the number of SH groups left to react with Ag gradually decreased to zero.

It is suggested that, in horse haemoglobin, 6 SH groups occur in two similar clusters of three and that the 3 groups within each cluster are close together.

Human oxyhaemoglobin in SDS solution has 8 SH groups which react with $AgNO_3$; it takes up 6 $HgCl_2$.

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The Deamination of Adenylic Acid (AMP-5) in Brain. By H. Weil-Malherbe. (*Runwell Hospital, Wickford, Essex*)

Muntz (1953) has shown that extracts of acetone-dried dog brain contain an adenylic deaminase requiring ATP as cofactor. Lyubimova & Matlina (1954) found that ATP and ADP activate the deaminase associated with myosin.

An enzyme has been prepared from aqueous extracts of acetone-dried ox brain and purified about 10–20-fold. The activity is associated with particulate matter; all attempts to solubilize the enzyme resulted in its inactivation. A slow deamination occurs with adenylic acid alone, although adenosine deaminase activity is absent; addition of ATP strongly activates the reaction. The preparation still possesses some ATPase and myokinase activity, but these activities are suppressed by omitting Mg^{2+} from the medium and by adding ethylenediaminetetraacetate.

The requirement for ATP is specific; ADP, ITP and IDP are inactive. Muntz (1953) found that ATP is recovered unchanged while adenylic acid is quantitatively converted into inosinic acid and ammonia. These observations have been confirmed.

The enzyme is inhibited in varying degree by all those bi- and tri-valent cations which have been tested with the exception of Ca^{2+}. It is also inhibited by phosphate and pyro-phosphate ions. It is not inhibited by the reaction products.

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The Formation of Dimethyl Selenide by Moulds in Presence of D- and L-Methionine or Thetins, All Containing the $^{14}CH_3$ Group. By P. B. Dransfield and F. Challenger (*Department of Organic Chemistry, The University, Leeds*)

The production of labelled trimethylarsine and dimethyl selenide by *Scopulariopsis brevicaulis* and of dimethyl selenide by *Aspergillus niger* was described recently (Challenger, Lisle & Dransfield, 1954). Choline chloride, betaine, DL-methionine labelled with [$^{14}CH_3$]- and [^{14}C]-sodium formate were added to the cultures. The trimethylarsine and dimethyl selenide (as mercurichlorides) were radioactive in each case, 95% of the methyl of the selenide being derived from methionine, from which the methyl group is probably transferred intact. The methylation percentage, M%, was much smaller with other sources and was calculated from the ratio

$$\frac{100}{n \times f} \times \frac{\text{radioactivity of methylated product/mole}}{\text{radioactivity of methyl source/mole}},$$

where n = number of methyl groups produced by methylation per molecule of product and f = the fraction of those labelled methyl groups or carbon atoms per molecule which are theoretically labile.

Further work shows that labelled D- and L-methionines are equally capable of furnishing a methyl group to selenium, again giving M%, 90–100. Labelled L-methionine plus an equal weight of non-radioactive D-isomer gave M%, 63. Labelled D-methionine plus non-radioactive L-isomer gave M%, 61 and 53. Dimethylacetothetin and dimethyl-β-propiothetin chlorides are poor methyl sources. Labelled with one $^{14}CH_3$ group they give an M% below 3; betaine gives 11–22. Our earlier work showed that the two thetins and betaine (unlabelled), separately added to *A. niger*–selenate cultures containing labelled DL-methionine, hardly affected the usual M%, 95, indicating that they are not direct methyl donors.

S - Methyl - DL - methioninesulphonium iodide, $^{14}CH_3.(Me).S^+(I^-).R$, gave M%, 36 in *A. niger* cultures, being twice as effective a methyl source as betaine. In admixture with labelled DL-methionine the unlabelled methiodide considerably reduced the usual M%, 95. This was presumably due to mobility of the methyl group of unlabelled methionine produced by loss of methyl from the methiodide, which is probably not a direct methyl donor. Liquid cultures of *A. niger* containing selenate and labelled methionine retain, when transmethylation ceases, 35% of the original radioactivity in the form of an acid containing sulphur (probably as $-SCH_3$), possibly nitrogen but no amino acid group or keto group. It is not $CH_3.S.CH_2.COOH$, $CH_3.S.CH_2.CH_2.COOH$ or a corresponding sulphone.

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Methyl Mercaptan in Relation to *Foetor Hepaticus*. By F. Challenger and J. M. Walshe. (*Department of Colour Chemistry, The University, Leeds and Medical Unit, University College Hospital Medical School, London*)

A woman, aged 45, entering hospital jaundiced and in light coma, had a strong foetor hepaticus in breath and urine and neurological signs of acute hepatic failure. Post-mortem, the liver weighed 840 g., owing to almost complete destruction of the parenchymal cells.

Nitrogen was passed through the urine, then through dilute sulphuric acid, (*a*) aqueous mercuric cyanide and (*b*) aqueous mercuric chloride. The deposit in (*a*) sintered from 150 to 180° (decomp.) and gave an odour of methyl mercaptan on acidification. Heating with water to hydrolyse any double compound $Hg(SCH_3)_2.Hg(CN)_2$, filtering and recrystallizing from ethanol, gave shining leaflets m.p. 170–174° and 168° when mixed with authentic $Hg(SCH_3)_2$, m.p. 167–168° (all with decomposition). The authentic sample still contained co-ordinated $Hg(CN)_2$, the correct m.p. being 174°.

Further confirmation was afforded by the unmistakable odour of dimethyl sulphide in the melting-point tube, due to the thermal decomposition $Hg(SCH_3)_2 = HgS + (CH_3)_2S$.

The deposit in (*b*) gave no odour with sodium hydroxide, indicating absence of $(CH_3)_2S$ from the urine. Dilute acid gave a strong odour of CH_3SH. It sintered at 150°, decomposing at 180°, behaviour consistent with that of a mixture of $CH_3.S.HgCl$ and its co-ordination compound with mercuric chloride. Deposit (*b*) arose either from CH_3SH passing through (*a*) or by fission of $CH_3.S.S.CH_3$ produced from mercaptan by oxidation.

Chromatographic examination of the plasma repeatedly showed moderate increases in common amino acids, but marked increase in methionine, suggesting that CH_3SH arises from this amino acid by a fission process resembling those observed by Challenger & Charlton (1947) with *Scopulariopsis brevicaulis* and by Binkley (1950) with thionase. Dent (1947) found increased excretion of α-aminobutyric acid after administration of methionine to a case of Fanconi syndrome. By transmethylation and re-methylation, often occurring in the liver, the methionine level in the body is kept constant. In severe liver damage transmethylation may be inhibited; consequently methionine may accumulate, as in this and other cases of severe parenchymal liver disease. Excessive doses of methionine produce toxic symptoms in man and rats (Barclay, Kenney & Cooke, 1945; Earle, Smull & Victor, 1942; Watson, 1949; Roth & Allison, 1950) and appear detrimental to moulds (Challenger, Lisle & Dransfield, 1954).

Possibly the high methionine concentration in the plasma aggravated the patient's condition.

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The Indirect Determination and Identification of Urinary 17-Hydroxy-20-oxosteroids Unsubstituted at $C_{(21)}$. By Joan I. Appleby and J. K. Norymberski.* (*Chemical Research Laboratory, Rheumatism Research Unit, Nether Edge Hospital, Sheffield*)

A recently communicated (Appleby, Gibson, Norymberski & Stubbs, 1954) method for the determination of 17-hydroxy-20-oxosteroids unsubstituted at $C_{(21)}$ (21-deoxy-17:20-ketols) consists in their selective conversion into 17-oxosteroids on consecutive treatment with sodium bismuthate, sodium borohydride, and sodium bismuthate, whereby all other ketones and ketogenic compounds present in the analysed mixture are converted into alcohols. The application of this method to urines was facilitated by the observation that the 21-deoxy-17:20-ketol grouping is resistant to acid treatment under conditions employed for the hydrolysis of urines. The now developed procedure is briefly as follows. The urine (usually 0·4 of a 24-hour specimen) is boiled for ten minutes with 10 vol. % of concentrated HCl, extracted with chloroform, the washed extract divided into two equal parts, one of which is submitted to the above outlined sequence of reactions; the other sample is used for a blank determination consisting of four reaction steps, the first of which is reduction with borohydride, the subsequent three steps being identical with those performed on the first sample; the formed 17-oxosteroids are measured as chromogens in the Zimmermann reaction.

3α:17α-Dihydroxypregnane-11:20-dione added to urines or to urine extracts was accounted for in the assay to the extent of 80 ± 10 %. So far, the urines of healthy persons and those suffering from rheumatoid arthritis have been found to contain less than 1 mg. of 21-deoxy-17:20-ketols in 24-hour specimens.

A pooled extract (approx. equivalent to 48-hour excretion) obtained from a boy with adrenogenital syndrome was treated as in the analytical procedure; the mixture of products was fractionated with Girard T reagent and the ketonic fraction chromatographed on alumina with gradient elution: 3α-hydroxytestan-17-one (aetiocholanolone) was isolated as the sole 17-oxosteroid. Consequently 3α:17α:dihydroxypregnan-20-one, the only likely precursor of aetiocholanolone in the employed sequence of reactions, is considered the sole 21-deoxy-17:20-ketol in the investigated urine.

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* Holder of an Empire Rheumatism Council Research Fellowship.

The Metabolism of the Porphyrin Precursor, δ-Aminolaevulic Acid, in Normal Man and in the Rat.

By N. I. Berlin, C. H. Gray, A. Neuberger and J. J. Scott. (*National Institute for Medical Research, Mill Hill, London, N.W. 7, and Department of Chemical Pathology, King's College Hospital, London*)

δ-Aminolaevulic acid (ALA) has been shown to be a precursor of porphyrins *in vitro* (Shemin & Russell, 1953; Neuberger & Scott, 1953; Dresel & Falk, 1953). In the present work, ^{15}N-labelled ALA was injected into rats; depending on the dose, 30–60% of the ^{15}N appeared in the urine within 24 hr. Of this, 13% was in the urea, 4% was in the form $^{15}NH_4$, and 83% was in the residual nitrogen. This latter fraction had the highest per cent excess ^{15}N; it contained no porphyrin, but much unchanged ALA and porphobilinogen (PBG), together with its linear polymer, porphobilin (Brockman & Gray, 1953). The per cent excess ^{15}N of the ammonia was higher than that of the urea; this is believed to be due to the formation of porphobilin, which involves loss of NH_3. Incorporation into haem was surprisingly low, and less than that resulting from a comparable dose of [^{15}N]glycine. The very slight extent to which nitrogen of ALA is converted back to glycine was demonstrated by isolation of [^{15}N]-hippuric acid after injection of benzoate. Up to 5% of injected ALA was converted to PBG, whose excretion was rapid and complete within 12 hr. Similar results were obtained on administering ALA to man; an additional interesting effect was the appearance of a transitory photosensitivity.

Employing ^{14}C-labelled ALA, the extent of incorporation of ALA, in normal man, into PBG, porphyrin, haem and stercobilin was measured, together with the expired $^{14}CO_2$. 74% of the total radioactivity was accounted for. After swallowing 1 g. of ALA (containing 5 μC. ^{14}C), the faeces of the following day contained the relatively enormous figure of 13 mg. of protoporphyrin of high specific activity, and a large quantity of stercobilin. The relative specific activities of ALA, PBG, porphyrin and stercobilin were in accord with the theory that each of these compounds is the precursor of the succeeding one, and that there is in the body a small pool of each. The dilution of stercobilin was relatively greater, as would be expected from normal haemoglobin breakdown. The haemin had by far the lowest activity. This may be due to the very rapid excretion of ALA (and also of PBG; Goldberg & Rimington, 1954). It is likely that porphyrin is convertible to bile pigment outside the haemopoietic system.

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A Method for the Separation of Riboflavin, Flavin Mononucleotide and Flavin-adenine Dinucleotide by Ionophoresis on Paper and its Application to the Determination of these Compounds in Micro-organisms. By J. L. Peel. (*Agricultural Research Council Unit for Microbiology, Department of Microbiology, Sheffield University*)

The ionophoresis of various flavin derivatives on paper has been studied using the apparatus of Markham & Smith (1952). A satisfactory separation of riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) is obtained in 2 hr. using 0·025 M ammonium formate–formic acid buffer pH 4·6 and a potential gradient of approx. 20 v/cm. (cf. Siliprandi, Siliprandi & Lis, 1954). Riboflavinyl glucoside (Whitby, 1952) has a mobility similar to that of riboflavin and the two cannot be separated easily. The amounts of riboflavin, FMN and FAD in a mixture can be estimated by working with 0·1–0·2 μmole total flavin and, after ionophoresis, eluting and measuring the light absorption at 450 mμ. of the separated flavins. This procedure gives a 90% recovery of total flavin.

For the estimation of flavins in micro-organisms, washed cells (1–5 g. dry wt.) are extracted with 10% (w/v) trichloroacetic acid (20 ml./g. dry wt.) for 10 min. at room temperature. Prior to the ionophoretic separation the flavins so extracted are partially purified by adsorption on Florisil (Floridin Company, Warren, Pennsylvania) followed by elution with 25% (v/v) aqueous pyridine and concentration by freeze-drying (cf. Dimant, Sanadi & Huennekens, 1952). With a mixture of flavins of known composition the combined extraction, purification and concentration procedure gave a recovery of 80%. The method has been applied to a variety of microorganisms and, with certain modifications, to animal and plant tissues.

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Citrate Accumulation from Glycollate by an Oxalate Decomposing Organism. By G. C. N. JAYASURIYA. (*Agricultural Research Council Unit for Microbiology, Department of Microbiology, University of Sheffield*)

The tricarboxylic acid cycle is the only reasonably well established pathway for terminal respiration in bacteria. Recently, however, evidence has accumulated which indicates that alternative pathways are operative for the oxidation of certain two-carbon compounds. The incubation of washed cells of an oxalate-decomposing organism with glycollate and monofluoroacetate results in the accumulation of citrate (Jayasuriya, 1954). This reaction involves the synthesis of citrate from a C_2 compound in the absence of any added C_4 acid. The mechanism of this reaction was further studied using [1-^{14}C]- and [2-^{14}C]-glycollate as substrates. The citrate formed was isolated chromatographically and the distribution of isotope determined.

The primary and secondary carboxyl groups of citrate were converted into CO_2 (Weinhouse, Medes & Floyd, 1946). The non-carboxyl carbon atoms were obtained as acetone by oxidation of citrate with dilute dichromate. The methyl groups of acetone were converted into iodoform which was then oxidized to CO_2. The carbonyl carbon of acetone appeared as the carboxyl group of acetate in the iodoform reaction and the acetate was decarboxylated with hydrazoic acid.

Only 7 % of the activity of the carboxyl group of glycollate is incorporated into citrate, the remainder being recovered in the respiratory CO_2. The specific activity of each carbon atom of the citrate is much lower than the specific activity of the respiratory CO_2. Most of the activity found in the citrate could be explained on the basis of known CO_2-fixation reactions.

On the other hand, 47 % of the activity of the α-carbon of glycollate is incorporated into citrate, the remainder being recovered in the respiratory CO_2. Here the specific activities of all six carbon atoms are of the same order of magnitude and are approximately 2·5 times the specific activity of the respiratory CO_2.

From these experiments it can be concluded that glycollate is converted into citrate. It is chiefly the α-carbon of glycollate that is incorporated into citrate, the carboxyl carbon being incorporated only to a small extent. It seems probable that the α-carbon of glycollate gives rise to a C_1 unit which is involved in the synthesis of citrate from glycollate.

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Aerobic and Anaerobic Synthesis of Fatty Acids in Mammary-gland Homogenates. By C. TERNER. (*National Institute for Research in Dairying, University of Reading*)

It is well known that mammary tissue is a site of active lipogenesis. While mammary slices from ruminants synthesize fatty acids from acetate alone, non-ruminant mammary tissue utilizes acetate only in the presence of glucose (Balmain, Folley & Glascock, 1952). The synthesis of fatty acids in cell-free preparations of rat and sheep mammary tissue was shown by Popják & Tietz (1954). Fatty acid synthesis sensitive to uncoupling agents such as *p*-nitrophenol (*p*NP) has now been observed in guinea pig mammary homogenates. These have already been shown to metabolize acetate, pyruvate and glucose (Terner, 1953, 1954).

The homogenates were incubated for 40 min. in the basal medium previously employed (Terner, 1954). When only [^{14}C]acetate was added, its incorporation into fatty acids was negligible. Fumarate ($1{\cdot}25 \times 10^{-3}$ M) caused marked stimulation which was accelerated by increasing the concentration of fumarate up to 0·005–0·01 M (rate of acetate incorporation 1–2 μmole/100 mg. dry wt./hr.). The addition of unlabelled glucose caused further stimulation and [^{14}C]glucose also supplied carbon for fatty acid synthesis, as originally shown in mammary slices by Balmain, Folley & Glascock (1952). Popják & Tietz (1954), however, found glucose to be ineffective in stimulating fatty acid synthesis from [^{14}C]acetate in their cell-free suspensions.

The incorporation of [^{14}C]acetate into fatty acids was strongly inhibited by *p*NP (2×10^{-4} M), fluoride (0·005–0·01 M) and arsenate (0·005–0·02 M). The inhibition by *p*NP was less complete in the presence of glucose. The uptake of inorganic phosphate, which can be observed in suitably reinforced mammary homogenates (Terner, 1954), was abolished by *p*NP concurrently with the inhibition of fatty acid synthesis. In this system fluoroacetate was also inhibitory. The results thus indicated that, while the synthesis of fatty acids from acetate depended mainly on its activation by phosphate bond energy

derived from Krebs-cycle oxidations, some activation could take place at the expense of glycolytic phosphorylations.

Anaerobic incorporation of [^{14}C]acetate into fatty acids has been observed under conditions promoting rapid glycolysis (Terner, 1952). The complete system included glucose, hexokinase and oxaloacetate. No synthesis was observable in the absence of hexokinase or oxaloacetate.

[^{14}C]pyruvate (plus fumarate) was readily utilized under aerobic conditions. In contrast to its inhibitory effect on the metabolism of acetate, *p*NP greatly increased the radioactivity of the respiratory CO_2 derived from [^{14}C]pyruvate, at the same time inhibiting fatty acid synthesis. In the absence of the inhibitor 30% of the pyruvate carbon appeared in the respiratory CO_2 and 70% was incorporated into fatty acids, while in the presence of *p*NP 90% of the pyruvate was oxidized to CO_2 and water. These findings support the conclusions drawn from previous studies of the effect of nitrophenols on the metabolism of pyruvate (Terner, 1953).

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The Site of an Adaptive (Induced) Enzyme System in Bacterial Extracts. By T. J. Bowen and S. Dagley. (*Department of Biochemistry, University of Leeds*)

Examination of bacterial cell-free extracts in the analytical ultracentrifuge has revealed components with sedimentation constants of approximately 40, 29, 20, 8 and 5 Svedberg units (Schachman, Pardee & Stanier, 1952; Siegel, Singer & Wildman, 1952; Billen & Volkin, 1954). Certain of these workers and Tissières (1954) have shown that the faster-moving particles are associated with various enzyme systems but positive correlation of enzyme activity with a single, separated component has not yet been reported.

Extracts of *Aerobacter aerogenes* capable of dissimilating citrate were prepared (Dagley & Dawes, 1953), freed from debris at 20000 *g* for 15 min. and on examination in the analytical cell showed the above-mentioned five peaks. Further centrifugation at an average field of 103000 *g* for 1 hr. gave a supernatant from which the top half was carefully removed and examined. The preparation contained almost all the citridesmolase activity and was free from the 40 and 29*S* peaks. Centrifugation at 150000 *g* for a further 2 hr. concentrated activity in the lower half of the tube: the upper supernatant possessed only weak activity and analysis revealed only the 8 and 5*S* peaks. The active fraction contained these peaks and in addition the third peak. When centrifuged at 187000 *g* in the analytical cell the total removal of this third peak was followed during the course of 90 min., the supernatant was carefully removed and the deposit taken up in a volume of phosphate buffer sufficient to refill the cell. This solution possessed almost the same activity as that given before the final centrifugation and the supernatant, after removal of the third peak, was almost devoid of activity.

Further work is in progress to decide whether the particles associated with citridesmolase activity are identical with those responsible for the 20*S* peak observed by other workers, or whether they are different particles sedimenting at a similar rate. The possible association of other adaptive enzymes with the third peak is under investigation.

This work arose during attempts to purify citridesmolase in collaboration with Dr E. A. Dawes.

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The Metabolism of the Sulphate Groups of Sulphated Polysaccharides and Mucopolysaccharides in Limb Atrophy in the Rat. By H. G. B. Slack. (*Rheumatism Research Centre, University of Manchester*)

The chondroitin sulphuric acid of rib cartilage of the adult rat has been shown by administration of $Na_2{}^{35}SO_4$ to have a half-life of approximately 17 days (Boström, 1952*a*). Furthermore it has been shown that the uptake *in vitro* of labelled sulphate into cartilage slices involves an esterification to chondroitin sulphate and is dependent upon the integrity of some enzyme system (Boström, 1952*b*). In previous experiments it was found that the synthesis of collagen apparently continues in atrophying hind

limbs of rats simultaneously with massive tissue wasting in these limbs (Slack, 1954). In view of the reported faster rate of metabolism of the sulphated polysaccharides in connective tissue as compared with collagen it seemed of interest to obtain some information about their metabolism under conditions of massive tissue wasting.

Two groups of adult rats, 12 in each group, were injected with 1 μc $Na_2{}^{35}SO_4$/g. body weight. One group were normal rats, the other rats with an atrophying right hind limb produced by operation six weeks previously. Half the rats of each group were killed 24 hr. after injection and the remainder 8 and 9 days after injection. Sulphate was recovered from three fractions of costal cartilage, and both normal and atrophying hind limb tissues, and its radioactivity measured as solid samples of $BaSO_4$. The three fractions were: (1) total water insoluble sulphate, (2) polysaccharide sulphate separated by digestion of the tissues with papain and subsequent purification by the method of Einbinder & Schubert (1950), and (3) sulphate remaining in the residue after the papain extraction.

In normal costal cartilage the loss of sulphate activity with time in the case of fraction 2 is in close agreement with the findings of Boström (1952*a*). Fraction 2 from the limbs of normal rats had a faster turnover rate than the chondroitin sulphate from costal cartilage, and in the atrophying hind limbs this papain-extracted fraction showed a greatly increased loss of sulphate activity with time. The initial radioactivities of fraction 1 samples are all higher than those of fraction 2 samples, the differences being especially marked in the limb extracts. Operation increases the rate of loss of activity with time in sulphate from all the tissues and in particular from the atrophying limb. By contrast residual sulphate, fraction 3, shows a slight gain in activity during the 8 days of the experiments in normal cartilage and limb, and a small loss from cartilage and limbs in operated animals.

The results suggest that some portion of the labelled sulphate is incorporated into polysaccharides other than chondroitin sulphate and having substantially different turnover rates from chondroitin sulphate.

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Tracer Studies with ^{131}I-Labelled Aryl Nitrogen Mustards in the Rat. By A. W. Craig and H. Jackson (introduced by L. P. Kendal). (*Christie Hospital, Manchester* 20)

Some aromatic nitrogen mustards show specific cytotoxic effects on dividing cells and have inhibitory powers on the growth of animal tumours (Haddow, Kon & Ross, 1948; Ross, 1953). They are much less potent and correspondingly less toxic than their aliphatic counterparts. Numerous aromatic nitrogen mustards have been synthesized (Ross, 1949; Everett & Ross, 1949) in attempts to find substances of greater potency but nothing appears to have been published concerning the metabolism of these substances.

In the present work *p*-iodo-*NN*-bis-(2-halogenoethyl) anilines labelled with ^{131}I were chosen as pattern substances. In all instances there was little evidence of retention of radioactive material in the tissues, in contrast to that found with ^{14}C labelled methyl bis-(2-chloroethyl)amine (Skipper, Bennett & Langham, 1951). A relatively high concentration of radioactive material was retained by the red cells, however, amounting to a few per cent of the dose administered. A similar effect has previously been reported after administration of labelled *p*-iodophenyl-urethane (Crick & Jackson, 1952) which was eventually traced to the formation of *p*-iodophenylhydroxylamine and its combination with haemoglobin (Crick & Jackson, 1953; Jackson & Thompson, 1954). Various labelled compounds of the type $I.C_6H_4.N(R_1R_2)$ have now been administered to rats and a similar selective localization occurred in the red cells.

Since an *in vitro* combination with red cells has only been demonstrated using *p*-iodophenylhydroxylamine or *p*-iodonitrosobenzene, it is suggested that the metabolic pathway of phenyl nitrogen mustards consists in part of degradation to these substances.

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Cofactors for the Enzymic Deamination of Histidine by Histidine α-Deaminase. By R. S. Edmundson and D. A. Hall. (*Department of Medicine, The University, Leeds*)

After treating a pig-liver extract successively with acidic and basic ion-exchange resins, the ammoniacal eluates from these possessed the power to activate liver histidine α-deaminase as measured by the spectrophotometric determination of the reaction product, urocanic acid (imidazole-4(5)-acrylic acid), and employing an apo-enzyme prepared by precipitating a liver extract to 30 % with acetone.

Of the two factors, A (the basic material from the Zeo-Carb resin) and B (acidic material from the Deacidite resin), the latter could also be prepared from a liver extract by precipitation as the barium salt and was finally purified by exchange chromatography on Dowex 1, either in the chloride or formate form.

Substance B is apparently a pyridine or pyridine-like nucleotide, being attacked by dithionite. Purine and pyrimidine nucleotides are ruled out on the grounds of their absorption spectra, and the former are also eliminated on the grounds of the comparative stability of the compound in acid solution. Perchloric acid hydrolyses the molecule to orthophosphate and an acidic fragment which reacts with CNBr and benzidine but giving only a very pale colour. Moreover, chromatographically the hydrolysis product behaves differently from nicotinamide or nicotinic acid. The carbohydrate moiety has been shown to contain five carbon atoms.

The second factor A has not yet been fully examined, but its activating power decreases on standing in acid or alkaline solution, and hence it is probably not a metallic cation, but an organic substance essentially of basic character, and which gives an insoluble phosphotungstate. Other evidence has been obtained which would seem to exclude the possibility of A being a metallic ion.

When the apo-enzyme is prepared by fractional extraction of liver, neither of the co-factor solutions (prepared from the resins) was active when tested separately, but together produced marked activation.

The evidence thus points to the deamination reaction as being quite distinct from the known oxidative, hydrolytic and trans-deamination processes which occur in liver.

DEMONSTRATION

A Vibratory-Shaker Warburg Apparatus. By H. Jackson and R. Thompson (introduced by L. P. Kendal). (*Christie Hospital, Manchester*, 20)

The apparatus consists of the conventional Warburg respirometers mounted separately on sheet Perspex ($19 \times 2 \times \frac{3}{8}$ in.). These slide into runners of the same material fixed to a single sheet mount (18×10 in.) also of Perspex, which accommodates six manometers. The lower edge of the mount is supported along the corresponding part of the bath, but at a distance of about $\frac{1}{4}$ in. from it. The upper edge is flush with the rim of the bath and approximately $\frac{1}{2}$ in. distant. Shaking is accomplished via a single brass rod bolted through a flange midway along the upper edge of the Perspex mount. The rod runs backwards across the top of the bath to the vibrator rod, to which it is attached by means of a clamp and boss. These latter enable the amplitude of the vibrations to be adjusted. Two small pads of sponge rubber are compressed between the upper corners of the mount and the bath, and smooth out the vibrations. The frequency used is 700/min. which affords efficient oxygenation, and agitation is not discontinued for the purpose of taking manometer readings. Apart from this, the apparatus is very simple to construct, is inexpensive and the vibrator and bath may be used for other laboratory purposes when the respirometers are not in use.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **333rd Meeting** *of the Biochemical Society was held at the National Institute for Medical Research, Mill Hill, London, N.W. 7., on Friday/Saturday, 15–16 October 1954, at 2.30 p.m., when the following papers were read:*

COMMUNICATIONS

Fluorimetric Determination of β-Glucuronidase. By J. A. R. Mead, J. N. Smith and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

Coumarin is metabolized in rabbits to 7-hydroxycoumarin (umbelliferone) (Mead *et al.* unpublished) which is readily detected by its intense fluorescence in ultraviolet light at pH 10. 4-Methylumbelliferone is equally fluorescent. *Umbelliferone glucuronide* (m.p. 150°; Found: C, 50·5; H, 4·7; H_2O, 5·1. $C_{15}H_{14}O_9$, H_2O requires C, 50·6; H, 4·5; H_2O, 5·1%) and *4-methylumbelliferone glucuronide* (m.p. 140°; Found: C, 46·8; H, 5·3; H_2O, 13·8. $C_{16}H_{16}O_9$, $3H_2O$ requires C, 47·3; H, 5·5; H_2O, 13·3%) were biosynthesized (rabbits) and found to be practically non-fluorescent when pure. The latter glucuronide is the more readily isolated and 4-methylumbelliferone is the cheaper. Umbelliferone and 4-methylumbelliferone can be accurately determined fluorimetrically in quantities of 0·01–0·02 μg./ml. or less.

Using these glucuronides the amount of β-glucuronidase in very small amounts of tissue can be determined directly. The method is many times more sensitive than the phenolphthalein glucuronide method. The tissue (10–30 mg.; much smaller quantities can be used) was homogenized in 3 ml. ice water and the mixture diluted with water to 10 or 100 ml. according to the amount of tissue and expected activity. 1 ml. of the diluted solution was incubated at 38° for 30 min. with 3·5 ml. 0·1 M acetate buffer pH 4·6 and 0·5 ml. 0·001 M glucuronide, with the relevant blanks. The incubated mixture was then diluted to 25 ml. with 0·1 M glycine buffer pH 10·3 (25 ml. cells were used) and the fluorescence measured in a Spekker fluorimeter against a quinine sulphate standard (4 μg./ml.).

Some results obtained, expressed as μg. 4-methylumbelliferone liberated/mg./hr. in acetate buffer pH 4·6, were as follows: rat: liver, 12·9; spleen, 11·8; skeletal muscle, 0·07; testis, 0·11; phrenic nerve, 0·53; guinea pig: liver, 4·3; spleen, 3·7; adrenals, 0·315; skeletal muscle, 0·0016. A sample of locust crop liquor (diluted 25 000 times for measurement) yielded 130 000 μg./ml./hr.

Potassium umbelliferone sulphate (Found: S, 11·3; K, 14·0. $C_9H_5O_6SK$ requires S, 11·4; K, 13·9%) and potassium *4-methylumbelliferone sulphate* (Found: S, 10·7; K, 13·3. $C_{10}H_7O_6SK$ requires S, 10·9; K, 13·3%) were also synthesized and had very weak fluorescences. These appear suitable for arylsulphatase assay.

The work was supported by a grant from the Medical Research Council.

The Metabolism of the Isomeric Trichlorobenzenes. By W. R. Jondorf, D. V. Parke and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

The trichlorobenzenes, particularly the 1:2:4-isomer, are breakdown products of the insecticide, benzenehexachloride ('gammexane') (cf. Luther, Koebel, Ruschenburg & Lampe, 1949), and the deleterious effects of crude benzenehexachloride on plants (e.g. wheat seedlings; Hocking, 1950) have been ascribed to the presence of trichlorobenzenes.

We have found that, in the rabbit, 1:2:3-, 1:2:4- and 1:3:5-trichlorobenzenes (0·5 g./kg. orally) are slowly metabolized to monophenols which are excreted conjugated mainly with glucuronic acid. The following conjugations (% of dose) were found during 5 days after dosing: 1:2:3- isomer, G, 50; E, 12; M, 0·3: 1:2:4-, G, 27; E, 11; M, 0·4: 1:3:5-, G, 21; E, 5; M, 0 (where G is glucuronide, E is ethereal sulphate, and M is mercapturic acid).

The major metabolite of the 1:2:3- isomer was 2:3:4-trichlorophenol (isolated; benzoate m.p. 141°); small amounts of 3:4:5-trichlorophenol and traces of 3:4:5-trichlorocatechol were detected by paper chromatography. The minute amount of mercapturic acid was not isolated as such but as a hexa-

chlorodiphenyldisulphide, m.p. 151°, not yet identified. The 1:2:4- isomer gave 2:4:5-trichlorophenol (benzoate, m.p. 92°) as the major metabolite, with small amounts of 2:3:5-trichlorophenol and 3:4:6-trichlorocatechol (paper chromatographic identification). 2:4:6-Trichlorophenol (benzoate, m.p. 74°) appeared to be the only metabolite of 1:3:5-trichlorobenzene.

This work was supported by a grant from the Agricultural Research Council.

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Vitamin D Balance Studies in Rats. By E. KODICEK. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

The paper-chromatographic technique for the estimation of vitamin D (Kodicek & Ashby, 1954) has been used to follow the fate of the vitamin *in vivo*. Thirty-six weanling rachitic rats were distributed between six groups and each rat was given by mouth with a micro-syringe a single dose of ergocalciferol, dissolved in 0·3 ml. arachis oil, in graded amounts according to the group, viz. 8, 4, 2, 1, 0·5 and 0·25 mg., respectively. On each of the two succeeding days the combined livers and faeces of three rats in each group were assayed chemically. The kidneys of rats that received 8 and 4 mg. ergocalciferol were also analysed.

On the first day after dosing, the total content of vitamin D per liver was, in decreasing order, for the individual groups 639, 540, 236, 140, 44 and 29 μg., respectively. The amounts consituted about 9–14 % of the dose, the rise in the liver reserves being directly proportional to the dose, except that at the 8 mg. level a relatively smaller rise occurred comparable to that found with large doses of vitamin A (Moore, Sharman & Ward, 1951). On the second day there was a significant drop in the liver reserves, as observed previously (Cruickshank, Kodicek & Armitage, 1953), to values of 543, 255, 135, 54, 32 and 14 μg. respectively. The decrease amounted to about 40–50 % of the amount present in the liver on the first day. The fate of the vitamin D that disappeared from the liver is at present unknown.

In the faeces, on the first day, the total vitamin D content for the individual groups was 2634, 812, 336, 198, 80 and 37 μg./day, respectively, amounting to 15–20 % of the dose. On the second day the excretion was 4–5 % of the dose.

In the kidneys, in the two groups examined, 18 μg. of vitamin D were found on the first day, and on the second, 15 and 6 μg.

The total amount of the dose recovered was of the order of 37 %. The recovery was significantly higher than that found previously (Cruickshank, *et al.*, 1953), mainly because of a higher recovery in the liver. It is concluded that the difference was caused by the different way of administration of vitamin D and the subsequent improved absorption of the dose.

The paper-chromatographic procedure allowed a simultaneous estimation of vitamin E in the liver, which was determined by the $FeCl_3$–dipyridyl method in eluates of chromatograms by Mr R. Ward to whom I am indebted.

The vitamin E content, in the form of α-tocopherol, was of the order of 3–10 μg. in the whole liver. The total liver vitamin A was 180–350 i.u.

I wish to thank Mr D. R. Ashby for his valuable technical assistance.

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The Isolation of a Fourth Kober-chromogen from the Urine of Pregnant Women. By G. F. MARRIAN and W. S. BAULD. (*Department of Biochemistry, University of Edinburgh*)

From crude crystalline oestriol separated from the ether-soluble 'strong phenolic' fraction obtained from acid-hydrolysed human pregnancy urine a new Kober-chromogen (KC-4), m.p. 277–280° (uncorr., evac. sealed capillary), $[\alpha]_D^{19} = +85°$ in ethanol, has been isolated in a yield of approx. 0·1 mg./l. urine. The methods employed for the isolation of KC-4 included countercurrent distribution between ether and an alkaline phosphate buffer (pH 12·0), and partition chromatography on Celite columns using the system 70 % methanol–ethylene dichloride.

KC-4 gave C and H values in close agreement with those required for a substance of formula $C_{18}H_{24}O_3$, and yielded a triacetate, m.p. 151–152° (uncorr.).

In the Kober reaction KC-4 developed maximal

colour with a shorter time of heating in the first stage of the reaction than is required for oestriol. Since oestriol may be dehydrated to oestrone in the first stage of the Kober reaction this finding suggested the possibility that KC-4 might be a stereoisomer of oestriol more sensitive to the dehydrating action of H_2SO_4 than the latter itself. By the preparation of an acetonide, m.p. 95° and 183–185° (uncorr.), it was shown that KC-4 contains a *cis* glycol grouping.

The identity of KC-4 with '*iso*oestriol A' (16-*epi*oestriol) prepared by Huffman and his co-workers (Huffman, 1942; Huffman & Darby, 1944; Huffman & Lott, 1949) from oestrone via 16-oximinooestrone and 16-oxooestradiol-17β was proved by mixed melting points of the triols and of their triacetates.

The possibility that KC-4 may be an artifact has not been disproved.

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Activation of Prostatic Acid Phosphatase. By GRACE M. JEFFREE. (*Pathology Research Laboratory, University of Bristol*)

Acid phosphatase activity in saline dilutions of stored seminal fluid was found to depend on the presence of soluble proteins or polypeptides, or of amino-compounds, particularly long-chain diamines or polyamines. Activity comparable to that obtained with serum derivatives (Jeffree, 1953) was found with high dilutions of gelatin, blood albumin, or protamines. Phosphatase values decrease extremely slowly with dilution of these activating substances, and a substrate containing 30 μg.% of gelatin still shows about half the activity of one containing 50 μg.%. This point of half activity was used as a convenient unit, 'A', for comparison of different activators; 'A' being defined as that concentration of any substance in the substrate which will produce half the acid phosphatase activity shown by the same dilution of prostatic phosphatase in a substrate containing 50 μg.% gelatin. For gelatin itself, 'A' is approximately 30 μg.%; for blood albumin 50 μg.%; for salmine sulphate 21 μg.% and for clupeine sulphate 20 μg.%. The protamines thus give slightly greater activation than gelatin, and albumin slightly less, since a low value of 'A' indicates high activity.

Similar activation was obtained with a number of amino-compounds, but much greater concentrations were required, particularly with short-chain diamines; activity being found in general to increase with the chain length between terminal amino-groups. 'A' for ethylamine is M/7; for ethylenediamine M/140; for hexamethylenediamine M/5200; for decamethylenediamine M/11 500, and for spermine M/30 000.

Activity is not decreased by the formation of benzyl or dinitrophenyl derivatives, or by treatment with formaldehyde; but completely destroyed by acetylation. Amides and α-amino acid groupings are inactive; histidine and tryptophan are inactive (to M/10 and M/100 respectively) whereas 'A' for both histamine and tryptamine is about M/200. Arginine shows slight activity, with 'A' about M/17, comparable to guanidine with 'A' about M/20. A trace of activity was found for ornithine; none for lysine, asparagine, citrulline, glycine or serine.

Activation by gelatin is not significantly reduced by peptic digestion, but is markedly reduced by digestion with trypsin or papain, and destroyed by hydrolysis with 8N-HCl. Thus activity decreases progressively with the breakdown of the peptide chain, suggesting a comparison with the amino-compounds, where high activity is associated with a long chain between terminal amino-groups.

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The Uptake of ^{32}P by the Nucleic Acids of Rabbit Tissues. By R. M. S. SMELLIE, G. F. HUMPHREY, E. R. M. KAY and J. N. DAVIDSON. (*Department of Biochemistry, The University of Glasgow*)

In a previous paper (Smellie, McIndoe, Logan, Davidson & Dawson, 1953) the course of incorporation of radioactive phosphorus into the deoxyribonucleic acid (DNA), nuclear ribonucleic acid (nRNA) and cytoplasmic ribonucleic acids (cRNA) of rabbit liver has been followed. In this communication a systematic comparison of the uptake of ^{32}P by DNA, nRNA and cRNA in rabbit appendix, bone-marrow, intestinal mucosa, kidney, spleen and thymus is reported. The results show that these tissues fall into three main groups: appendix and bone-marrow in which DNA is rapidly renewed,

kidney where the rate of renewal of DNA is very low, and intestinal mucosa, spleen and thymus in which the renewal rate of DNA is intermediate between that of the two other groups. In all the tissues examined the rate of ^{32}P uptake by nRNA is considerably higher than that for DNA or cRNA. The effect of X-irradiation and of phenylhydrazine induced haemolytic anaemia (with resultant hyperplasia of the bone marrow) on the incorporation of ^{32}P into the nucleic acids of different tissues has also been examined. Irradiation of tissues which are normally proliferating rapidly causes a marked depression in the uptake of the isotope by DNA, nRNA and cRNA. Phenylhydrazine treatment brings about a marked increase in the incorporation of ^{32}P into the DNA of bone marrow and spleen with smaller effects on the cRNA of these tissues. These observations provide supports for the view that there is a correlation between DNA turnover and the rate of cell division in different tissues as reported by Stevens, Daoust & Leblond (1953) and Daoust, Bertalanffy & Leblond (1954).

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A Chromoprotein from the Eggs of *Pomacea canaliculata*. By D. F. CHEESMAN. (*Department of Physiology, Bedford College, University of London*)

The eggs of the South American water snail *Pomacea canaliculata australis* (Orb.) have a vivid rose-red pigmentation, which has been shown by Comfort (1947; 1949) to be a carotenoid-pigment complex. A homogenate of the eggs in water shows light absorption maxima at 426, 510 and 545 mμ. and an inflexion in the absorption curve at 480 mμ. The spectrum resembles that of many carotenoids in organic solvents. The pigment has been isolated by a method similar to that used by Kuhn & Sörensen (1938) for the ovoverdin of lobster eggs.

The pigment is adsorbed by $Al(OH)_3.C\gamma$ at pH 5·0 and eluted by 0·2M-Na_2HPO_4. Two repetitions of the adsorption-desorption cycle eliminate the maximum at 426 mμ. from the spectrum, which contains a 'protein' peak at 280 mμ. Further adsorptions produce no quantitative changes in the spectrum, the quotient $E_{280\ m\mu}/E_{510\ m\mu}$ remaining constant at 3·8. This suggests that the main pigment, apparently a chromoprotein with a carotenoid prosthetic group, has been isolated in a fairly pure form. The final spectrum shows absorption maxima at 280, 510 and 545 mμ. and inflexions at 330 and 480 mμ.

Treatment of a solution of the pigment with acetone under anaerobic conditions and extraction with petroleum ether yields a carotenoid, predominantly hypophasic in the system petroleum ether–90% (v/v) methanol. This has a single-banded spectrum with a maximum at about 472 mμ. in petroleum ether (b.p. 60–80° C.) and 493 mμ. in pyridine. In these properties, and its chromatographic behaviour on $CaCO_3$, it closely resembles astacene prepared from lobster hypodermis. Until the carotenoid has been crystallized, however, its identity with astacene must remain in doubt.

Dry-weight determinations on dialysed solutions, together with spectrometric determinations of the extracted carotenoid, give an apparent molecular weight for the chromoprotein of about 300 000, when it is assumed that the prosthetic group is astacene, for which the molar extinction values have been recorded by Kuhn & Lederer (1933), and that one carotenoid molecule is bound to each molecule of protein.

The chromoprotein is precipitated by full saturation of its solution with Am_2SO_4. It is coagulated very slowly by heat. When a solution at pH 5 is heated at 100° for 1 hr. the protein remaining in solution has an unaltered absorption spectrum. Heating at pH 8·4 for 1 hr. gives little or no precipitation, but the spectrum in the visible region becomes less complex, finally showing a single maximum at about 475 mμ. This change is brought about in the cold at pH 14, and is apparently associated with a 'denaturation' of the protein, in that the latter is sensitized to heat coagulation at lower pH values and spreads more readily to an unimolecular film at a water surface. The denatured protein shows a flocculation maximum at pH 4·8, which presumably represents its isoelectric point.

The protein is soluble in 60% (v/v) ethanol or *iso*propanol, in which it shows the single-banded spectrum and from which it spreads rapidly on surfaces to give unimolecular films.

The results are consistent with the view that the carotenoid stabilizes the protein in its native configuration.

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The Role of Fumarate and Pyridine Nucleotides in the 11β-Hydroxylation of 11-Deoxycorticosterone (DOC). By A. C. BROWNIE and J. K. GRANT. (*Biochemistry Department, University of Edinburgh*)

Hayano & Dorfman (1953) claim 'specificity and absolute necessity for fumarate' for the 11β-hydroxylation of DOC by saline-washed residues of ox-adrenal homogenates. Brownie & Grant (1954) however obtained hydroxylation in the presence of α-oxoglutarate, with fumarate formation inhibited by malonate, using ox-adrenocortical mitochondria prepared in sucrose.

It has now been shown that disorganization of the mitochondrial enzymes by acetone drying or pre-incubation in hypotonic media results in the development of a specific requirement for fumarate for DOC 11β-hydroxylation. Acetone-dried preparations require fumarate + TPN (or DPN + ATP) (cf. Hayano, 1954). Pre-incubated preparations have similar requirements but cannot use DPN + ATP. Succinate and L-malate show some effect in both preparations but much less than fumarate. Malonate inhibits hydroxylation in the presence of TPN and fumarate, suggesting that reduction of the fumarate is involved, possibly by a reaction of the type $TPNH + \text{fumarate} + H^+ \rightarrow TPN^+ + \text{succinate}$ suggested by Slater (1949) for DPNH.

It is not yet possible to decide on the primary hydrogen acceptor for 11β-hydroxylation under these conditions since no hydroxylation was obtained with large (non-catalytic) amounts of either TPN or fumarate alone. Nor was it found possible to replace either TPN or fumarate by pyocyanine, ferricyanide, methylene blue, brilliant cresyl blue or 1:6-dichlorophenolindophenol. The last was added slowly to minimize possible inhibition (Drysdale & Lardy, 1953).

Since the analytical methods employed in these experiments would not distinguish 9:11-anhydrocorticosterone from DOC the formation of this hypothetical unsaturated intermediate (Hayano, Dorfman & Yamada, 1951) in the presence of non-catalytic amounts of TPN and without fumarate cannot be discounted.

It is emphasized however that the demonstration of 11β-hydroxylation of DOC by intact mitochondria in the absence of fumarate casts doubts on the significance of observations with damaged enzyme preparations which require fumarate.

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The Fractionation of Rabbit γ-Globulin by Partition Chromatography. By R. R. PORTER. (*National Institute for Medical Research, London, N.W. 7*)

In the rabbit, antibodies are confined to the γ-globulin fraction and may be separated from each other and from the inert γ-globulin by combination with the specific antigen. Yet no chemical or physical distinction has been found between antibodies and the inert γ-globulin. An attempt has been made to resolve normal and immune rabbit γ-globulin by partition chromatography (for details of the technique see Porter, 1954).

It was found that the globulin would partition, without denaturation, and was sufficiently soluble in the liquid phases produced by the mixture 480 g. water, 238 g. 2·5M potassium phosphate pH 9·2 and 312 g. cellosolve (3 parts by volume of butyl to 2 parts of ethyl) at $-3{\cdot}1 \pm 0{\cdot}05°$. The partition ratio is 8/1 in favour of the aqueous phase. This phase which was, therefore, the stationary phase was held by twice its weight of Celite 545 (a large-particle kieselguhr). Successful chromatography was only possible over a very narrow range of concentration of the components of the mixture.

When chromatographed using this phase mixture, γ-globulin from stock rabbits showed one very spread peak. However, when samples from individual fractions were re-run on a smaller column using the same phase mixture a discrete peak was obtained with an R value corresponding to that of the fraction used. This suggests that the chromatography is satisfactory and that the γ-globulin is a complex mixture of very similar components. The recovery of the protein from the chromatogram is about 80% and rises to 95% in a phase mixture containing more water, when the peak is less spread.

When γ-globulin prepared from the sera of rabbits immunized against alum-precipitated ovalbumin was examined it was found that the composition varied with the stage of immunization of the rabbits. In the early stages there was one antibody peak con-

fined to the slower running part of the globulin, more than half the globulin being free of antibody. Later a second chromatographically distinct antibody fraction appeared with a higher R value. Later still this became the main fraction, but the slower running antibody fraction was still present.

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The Toxic Principle in Seeds of *Dichapetalum toxicarium* (Ratsbane). By R. A. Peters,* R. W. Wakelin, F. T. Birks, A. J. P. Martin and Joan Webb. (*Department of Biochemistry, University of Oxford, A.E.R.E., Harwell, and National Institute for Medical Research*)

Though fluoroacetic acid can be detected in extracts from the seeds proper, unlike the South African *Dichapetalum cymosum* the main toxicity resides in a petrol-ether-soluble fraction, and withstands saponification. The K salts are toxic to rats in doses of approximately 100 mg./kg. by intraperitoneal injection, thereby causing large accumulations of citric acid, especially in heart. The presence of CF was detected in a concentration of $0{\cdot}14 \pm 0{\cdot}06$ % F (Birks, 1951; Gillieson & Newcombe, 1951) by the spectro-chemical method, bands 7128·0 and 7202·4 Å. As attempts to fractionate the mixed soaps with solvents were unsuccessful, the mixed fatty acids were fractionated by reversed phase partition chromatography (Howard & Martin, 1950). In this way from 11 mg. approximately 400 μg. was separated in the long-chain fraction, 1·25 μg. of which was enough to cause accumulation of citric acid from fumarate in the kidney particle test. The F content was 6·7 %. By the use of malonate blocking, it was shown that the jamming of citric acid metabolism *in vitro* involved synthesis; hence, it seems likely that fluorocitric acid is synthesized as in the case of fluoroacetate from active fluoroacetyl groups supplied from the fluorofatty acid, possibly by some of the enzymes recently described by Lynen & Ochoa (1953) and by Green and colleagues (1954.)

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This is now a second example in nature of the existence of a CF-containing substance, and the first of a fluorinated fatty acid. These observations suggest the hypothesis that the paralysis and sensory disturbances in human 'broke back' induced by eating these seeds (Renner, 1904) is due to the 'lethal' synthesis from a fluorinated fatty acid of fluorocitric acid which then blocks metabolism at the aconitase stage of the citric cycle. So changes in this seem directly related to clinical dysfunction in the central nervous system. It is strange that the plant should tuck away a fluorofragment in one fatty acid rather than in the fats generally.

We are indebted to Sir John Simonsen, to Dr Galley and to those in Sierra Leone for their co-operation in providing the seeds.

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The Deiodination of Thyroxine and Triiodothyronine *in vitro*. By W. E. Sprott and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, London, S.W.* 1)

Following the recent discovery of triiodothyronine (Gross & Pitt-Rivers, 1952; Roche, Lissitzky & Michel, 1952) the importance of deiodination processes has been widely recognized but little *in vitro* evidence for the deiodination of thyroxine has been produced. We have studied the output of iodide from systems incorporating either radioactive or non-radioactive thyroxine in homogenates of various rat tissues, and have demonstrated active deiodination by both methods. The iodide was estimated after precipitation of proteins by 5% trichloroacetic acid, either by electrometric titration (Wilkinson, Sprott, Bowden & Maclagan, 1954) or by counting in the Veall (1948) liquid Geiger-Müller counter.

In the radioactive (R) experiments 4 μg. of thyroxine labelled with ^{131}I in the 3′5′ position and 1 g. of rat liver was used in each system, and up to 70 %

deiodination was obtained in 3 hr. (expressed as percentage of thyroxine radioactivity). *R* systems had a broad pH optimum between pH 5 and 7, and were inhibited by exclusion of air, by cyanide, citrate and by two typical antithyroxine substances. Similar results were obtained with homogenates from kidney, brain, muscle, spleen, adrenal thyroid and intestinal mucosa.

In non-radioactive (*N*) systems 5 mg. of L-thyroxine per g. of liver was employed and deiodination of up to 20 % was observed at pH 3·5 and at pH 9·5 in 18 hr. (expressed as percentage substrate iodine in 3′5′ positions). L-Triiodothyronine was also attacked at pH 9·5. This system was inhibited by exclusion of air and by cyanide but not by citrate or by antithyroxine substances. Both systems were resistant to heat, requiring 2 hr. at 100° for complete inactivation. The other products of the reaction were not identified with certainty but some chromatographic evidence for the production and subsequent destruction of triiodothyronine was obtained in the *R* liver systems.

The results indicate the presence of enzyme systems in rat tissues which actively deiodinate thyroxine and triiodothyronine, and support our theories of the mode of action of antithyroxine substances (Maclagan, Sprott & Wilkinson, 1952) which appear to act by inhibiting these deiodination processes.

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The Metabolism of δ-Aminolaevulic Acid in Acute Porphyria. By A. Neuberger, J. J. Scott and C. H. Gray. (*National Institute for Medical Research, Mill Hill, London, N.W. 7, and King's College Hospital Medical School, Denmark Hill, London, S.E. 5*)

The metabolism of δ-aminolaevulic acid (ALA) in a normal man has already been studied (Berlin, Gray, Neuberger & Scott, 1954). ALA has now been administered to two patients with acute porphyria. Case I (f.) was in severe relapse; case II (m.) was in moderate remission. Basal urinary porphobilinogen (PBG) excretion was measured before administration of the labelled compounds; there was no ALA detectable in the urine of either patient before administration of this compound. ^{14}C-labelled ALA was given orally to case I; the temporarily increased rate of PBG excretion which resulted was measured and the specific activity of ^{14}C-labelled PBG isolated from the urine was determined. There was no porphyrin present in the freshly voided urine. [^{15}N]-ALA and ^{14}C-labelled PBG were administered simultaneously to case II by duodenal tube. This procedure prevents destruction of the PBG by the gastric acid. The increased rate of PBG excretion was estimated as before and the quantity of ^{14}C and ^{15}N in the PBG was determined. In the second experiment, [^{15}N]PBG alone was administered to case II. Comparison of the data with those obtained when ^{14}C-labelled ALA was administered to a normal male, permit the following deductions to be made. The percentage of administered ALA converted to urinary PBG in acute porphyria is many times greater than in the normal subject. The observed dilutions of isotope will be compared with those found in a normal subject, and the aetiology of acute porphyria will be discussed in the light of these differences.

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The Enzymic Conversion of δ-Aminolaevulic Acid to Porphobilinogen. By K. D. Gibson, A. Neuberger and J. J. Scott. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

δ-Aminolaevulic acid (ALA) is a precursor of porphyrins (Neuberger & Scott, 1953; Dresel & Falk, 1953) and of haem (Shemin & Russell, 1953). Porphobilinogen (PBG), which is probably formed as an intermediate, has been shown to give rise to porphyrins in avian red cells (Falk, Dresel & Rimington, 1953) and in green algae (Bogorad & Granick, 1953). This communication describes some properties of

an enzyme catalysing the condensation of ALA to PBG, which we have tentatively named δ-aminolaevulic acid dehydrase.

Estimation of enzyme activity is carried out *in vacuo*; the reaction then does not proceed beyond the formation of PBG. To avoid loss of PBG at high or low pH values, protein is removed by precipitation with colloidal Fe_2O_3, the colloid being discharged by addition of $CuSO_4$. The enzyme has been found in fresh and acetone-dried rabbit liver, rabbit kidney, avian red cells (cf. Dresel & Falk, 1953), *Corynebacterium diphtheriae*, *Bacterium cadaveris*, and acetone-dried ox liver. No activity was detected in two preparations of acetone-dried yeast.

Aqueous extracts of an acetone powder of ox liver have been used as the source of enzyme for purification. By the use of heat, precipitation with $(NH_4)_2SO_4$, and adsorption on to $Ca_3(PO_4)_2$ gel, a purification of about 150-fold has been achieved. The most active preparations obtained so far catalyse the formation of about 40 μmoles of PBG per hour per mg. protein, at pH 6·8 and 38°. In a large-scale experiment in which 53 mg. PBG were formed from ALA in the presence of the enzyme, 20 mg. of crystalline material were isolated by the method of Cookson & Rimington (1954). The chromatographic behaviour of this material in butanol–acetic acid (Campbell, Work & Mellanby, 1951) was identical with that of PBG isolated from the urine of rabbits and of humans.

Evidence has been obtained that δ-aminolaevulic acid dehydrase is a sulphydryl enzyme. Purified preparations require to be activated with glutathione, cysteine being about half as effective. Thioglycollic acid, ascorbic acid and $Na_2S_2O_4$ do not activate. The activity of a heat-purified extract is completely inhibited by 10^{-3} M iodoacetamide and by 10^{-3} M *p*-chloromercuribenzoate, the effect of the latter being largely reversed by the addition of an equimolar amount of glutathione. The enzyme is also inhibited completely by 3×10^{-3} M disodium ethylenediaminetetraacetate. No Ehrlich-positive compound is formed when purified δ-aminolaevulic acid dehydrase is incubated with ε-amino-δ-oxocaproic acid or with α, δ-diamino-γ-oxovaleric acid.

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The Effects of Synthetic Fluoro Compounds on the Metabolism of Acetate and Citrate. By E. M. Gal,* R. A. Peters† and R. W. Wakelin. (*Department of Biochemistry, University of Oxford*)

Intraperitoneal injection of fluorocitrate (synthetic, Rivett (1953) or enzymic) or triethyl ester produced rapid and large citrate accumulation in liver as well as those noted in brain (rats), suggesting that accumulations where found sporadically in liver with fluoroacetate (Potter & Busch, 1951; Ord & Stocken, 1953) are due to penetration of fluorocitrate synthesized elsewhere. Fluoroacetamide (10 mg./kg.) fluoroacetylhydroxamic acid (8 mg./kg.) produced no 'liver' citrate increases. Diethylfluorooxaloacetate (15 mg./kg. intraperitoneal) caused slight increases in kidneys and fluoromalamide (300 mg./kg.), methylfluoromalate (150 mg./kg.; Taylor & Kent, 1954) and fluoropyruvate (80 mg./kg.) gave none. The latter in doses of 80 mg./kg gave convulsions (non-lethal) in 4 hr., but caused no rise in blood pyruvate, though *in vitro* fluoropyruvate gave irreversible inhibition of yeast carboxylase, of pyruvate oxidation (brain) and of butyrate condensation to citrate but not of citrate disappearance (kidney particles).

In male albino rats dosed with fluoroacetate (5 mg./kg.), fluorocitrate (synthetic 40 mg./kg.) and fluoropyruvate (80 mg./kg.) and injected intraperitoneally with 5 mg./kg. of [*Methyl*-^{14}C]acetate 30 min. later, expiratory CO_2 (4 hr.) showed 40, 30 and 50 % of the total activity injected against 75 % for controls. The organs and carcass showed higher radioactivity in the poisoned animals. Homogenates of the livers and kidneys of fluoroacetate and fluorocitrate poisoned rats given labelled acetate showed more activity in the supernatant than in the particles. The citrate level in the kidney was highest in the supernatant whereas that of the liver in the particles. Hence as indicated already *in vitro* fluoroacetate and acetate activation in the organs differ. Fluorocitrate must interfere with fat metabolism *in vivo*, because it leads to rapid and marked urinary

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appearance of ketone bodies. Unlike fluoroacetate, intraperitoneal fluorocitrate (20 mg./kg.) (synthetic) though increasing citrate in brain, produced no convulsions in 2 hr. Differing from acetylhydroxamic acid (15 mg./kg.) intraperitoneal fluoroacetylhydroxamic acid (8 mg./kg.) gave convulsions in 1½ hr., with slight increased kidney citrate. Extending earlier results on other animals (Peters, Wakelin & Hastings, 1953) intracerebral injections in rats of 20 μg./kg fluorocitrate produced convulsions, whereas 200 μg./kg. fluoroacetate did not. Convulsions caused by fluoropyruvate and fluoroacetylhydroxamate had a different origin.

We thank Mr R. Brown for fluoropyruvic acid.

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Metabolism of Pyruvate by Washed Suspensions of *Bacillus subtilis*. By C. E. Sewell and H. K. King. (*Biochemistry Department, Johnston Laboratories, University of Liverpool*)

While studying the synthesis of alanine by reductive amination of pyruvate (Fairhurst, King & Sewell, 1952) we have investigated the metabolic pathways of pyruvate in *Bacillus subtilis*. Washed suspensions of the organism were incubated with pyruvate, both in the presence and absence of ammonia, at pH 7 anaerobically. The lactate, acetate, formate, carbon dioxide and alanine formed were assayed and accounted for approx. 95 % of the pyruvate utilized. Changes in the carbon content of the cells themselves were slight, and no detectable amounts of succinate, acetylmethylcarbinol, or neutral volatile compounds were formed.

In the absence of ammonia reactions (1)–(3) account for nearly all the pyruvate used:

(1) $CH_3.CO.COOH + 2H \rightarrow CH_3.CHOH.COOH$,

(2) $CH_3.CO.COOH \rightarrow CH_3.COOH + CO_2 + 2H$,

(3) $CH_3.CO.COOH \rightarrow CH_3.COOH + HCOOH$.

If ammonia is present, about a third of the pyruvate is converted into alanine:

(4) $CH_3.CO.COOH + NH_3 + 2H \rightarrow CH_3.CH(NH_2)COOH$,

whilst the reductive reaction (1) is largely suppressed. A new reaction appears however, since the acetate now produced far exceeds the CO_2 (reaction (2)) plus the formate (reaction (3)). The overall equation for this reaction was found to be:

(5) $2CH_3.CO.COOH + 4H \rightarrow 3CH_3.COOH$,

which could be arrived at by the Thunberg-Knoop dicarboxylic acid cycle, the carbon dioxide for the first stage being supplied by reaction (2);

Pyruvate + CO_2 → oxaloacetate → malate → fumarate → succinate → 2 acetate.

In the presence of malonate (0·004 M), inhibiting the cycle at the fumarate → succinate stage, reaction (5) was suppressed. The synthesis of alanine was not affected however, and no suggestion is offered as to why the presence of ammonia should lead to reaction (5) taking place.

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Metabolic Activity of Blood Cells Studied with ^{14}C. By R. J. Robinson and R. V. Coxon. (*University Laboratory of Physiology, Oxford*)

As a preliminary to some experiments upon the formation of $^{14}CO_2$ from labelled intermediary metabolites in the whole animal we have investigated the extent to which $^{14}CO_2$ is produced from glucose and certain of its derivatives by the animal's blood.

For this purpose freshly drawn heparinized blood was incubated with the labelled substrate in gas-tight syringes for 1 hr. at 37°. The blood was then analysed for total CO_2 content in the Van Slyke manometric apparatus and the radioactivity of the liberated gas determined by the method of Van Slyke, Steele & Plazin (1951). In this way oxidation of the carbon of uniformly-tagged glucose and of carbonyl-labelled pyruvate was shown to take place in whole blood from cats, dogs and rabbits. The amount of radioactive CO_2 found corresponded to the complete oxidation of about 0·1 mg. glucose/100 ml. blood/hour. Production of $^{14}CO_2$ did not

occur if the blood was boiled before incubation or kept in ice instead of at 37°, indicating an enzymic process.

Further experiments using selective inhibitors led to the finding that 0·001 M arsenite, whereas it depressed the production of CO_2 from pyruvate by 90%, did not affect that from glucose. Fluoride (0·24 M) inhibited the oxidation of pyruvate by 95% but the oxidation of glucose was reduced by only 60%. 0·004 M malonate, which, according to Pardee & Potter (1949), should inhibit oxidation of both succinate and oxaloacetate almost completely, produced only a 12% reduction in the oxidation of both pyruvate and glucose in our system.

These results suggest that a pathway other than that involving the glycolytic sequence plus the tricarboxylic acid cycle may be operative in the blood.

Oxidative activity is absent from plasma and an attempt has been made to determine which sections of the cell population possess it. By single or repeated centrifugations preparations were obtained in which the proportion of red to white cells was altered from that in whole blood. It then became apparent that the activity per cell was much greater among the leucocytes than among the erythrocytes by a factor of around 10^4 and later experiments have suggested that the oxidative activity of whole blood derives almost exclusively from the former.

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The Distribution of Radioactivity in Crystalline β-Lactoglobulin Following the Injection of [^{14}C]Valine and Lysine into a Goat. By B. A. Askonas, P. N. Campbell, C. Godin and T. S. Work. (*National Institute for Medical Research, London, N.W.* 7)

It has been reported previously that [^{14}C]amino acids isolated from different peptide sequences of casein had the same specific activities 4 hr. after the injection of the labelled amino acids into a goat (Askonas, Campbell & Work, 1954).

This work has now been extended to the isolation of [^{14}C]lysine and valine from different parts of an electrophoretically homogeneous protein, i.e. crystalline goat β-lactoglobulin. In addition the time interval between injection of the radioactive amino acids and collection of milk has been shortened to reduce the effects of equilibration between the pools of peptides and free amino acids.

Milk was collected 2 hr. after the intravenous injection of DL-[^{14}C]lysine-HCl and DL-[^{14}C]valine into a goat and β-lactoglobulin was crystallized (Askonas, 1954). Methods used for hydrolysis and fractionation of the peptide mixture were essentially those described previously (Askonas *et al* 1954), although fractionation on the ion-exchange column was greatly improved by the use of a new resin, Zeokarb 225 W.R. 1:1 200–400 mesh (Campbell & Work, 1954). Eleven di- and tri-peptides containing lysine and six peptides containing valine residues were isolated and the specific activities of the dinitrophenyl amino acids were determined. Five out of the six valine residues had activities between 2057 and 2190 cts./min./cm.^{2}. These results lie within 3% of the value obtained for valine isolated from a complete hydrolysate of casein. One valine residue had an activity of 1030, 50% lower than the mean value. The isolated lysine residues gave counts of 2460, 2960, 2250, 1800, 2620, 2480, 2120, 2100, 2215, 2220, 2480 and 2080, with a value of 2300 for the lysine isolated from a complete hydrolysate of the protein. These values are within the experimental error of the method.

Thus except for one valine residue, the lysine and valine isolated from different peptide sequences in lactoglobulin had substantially the same radioactivity as the corresponding amino acid obtained from a complete hydrolysate of β-lactoglobulin. No evidence for bound amino acid intermediaries could be found, and no peptides from blood proteins appear to be utilized for the synthesis of β-lactoglobulin. These results are contrary to the findings of Steinberg & Anfinsen (1952) using an *in vivo* system. These authors detected significant differences in the radioactivity of the different peptide fractions obtained from ovalbumin.

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Unspecific Hydroxylation Reactions in the Metabolism of Aromatic Amino Acids. By C. E. DALGLIESH. (*Postgraduate Medical School, Ducane Road, London, W.* 12)

Metabolism of the aromatic amino acids involves hydroxylations (e.g. phenylalanine → tyrosine; kynurenine → 3-hydroxykynurenine) brought about by highly specific enzymes. Hydroxylation of foreign aromatic compounds in the body must be brought about either non-enzymically or by much less specific enzymes. In the absence of the specific hydroxylating systems, due to a congenital or an induced defect, the normal hydroxylated metabolites of the aromatic amino acids might still be formed by these non-specific pathways. The recent development (Udenfriend, Clark, Axelrod & Brodie, 1954; Brodie, Axelrod, Shore & Udenfriend, 1954) of a simple system (Fe^{2+}, ascorbic acid, ethylenediaminetetraacetic acid and oxygen; 37°; pH 6·7) which carries out hydroxylation of aromatic compounds in a manner shown to be closely analogous to *in vivo* hydroxylations (Axelrod, Udenfriend & Brodie, 1954) allows this possibility to be investigated.

This hydroxylating system has been applied to several substances, and the products identified chromatographically. Products marked with an asterisk have been compared with authentic materials; structures of the remainder are inferred. The following reactions are readily brought about; tryptophan → 5-hydroxytryptophan* and 7-hydroxytryptophan; kynurenine → 3-hydroxykynurenine* and 5-hydroxykynurenine*; anthranilic acid → 3-hydroxyanthranilic acid* and 5-hydroxyanthranilic acid*; indoleacetic acid → 5-hydroxyindoleacetic acid* and 7-hydroxyindoleacetic acid; phenylalanine → tyrosine*, *o*-tyrosine*, also 2:5-dihydroxyphenylalanine* (2:5-dopa), 3:4-dopa* and 2:3-dopa* and, probably, hydroquinone and/or catechol and alanine; tyrosine → 3:4-dopa* and (?) hydroquinone; *o*-tyrosine → 2:3-dopa*, 2:5-dopa* and (?) catechol; phenylacetic acid gives hydroxy- and dihydroxy-derivatives of as yet undetermined orientation.

It is therefore considered that two routes exist for the hydroxylations in aromatic amino acid metabolism, the one specific, the other non-specific and presumably much slower. The wide variety of metabolites excreted by the phenylketonuric (Boscott & Bickel, 1953) may be due to action of the non-specific hydroxylating system on products accumulated as a result of lack of the specific system converting phenylalanine into tyrosine, rather than to the existence of several metabolic blocks. 'Unspecific' hydroxylation as a source of part of the 'leakage' of phenylalanine to tyrosine shown isotopically in a phenylketonuric (Udenfriend & Bessman, 1953) should be reconsidered. 'Unspecific' hydroxylation may cause confusion in attempts to isolate the labile specific hydroxylating systems involved in aromatic amino acid metabolism. It may also be concerned in melanogenesis.

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Effects of Methylbromide, Ethylenedibromide, and Ethylenedichloride on the Phosphorus Metabolism of *Musca domestica* L. By F. P. W. WINTERINGHAM and G. C. HELLYER. (*Department of Scientific and Industrial Research, Pest Infestation Laboratory, Slough, Bucks*)

The distributions of ^{32}P-labelled intermediates extracted from the thoracic muscle of normal and poisoned adult houseflies have been compared quantitatively by a combined radioactive tracer-paper chromatographic technique (Winteringham, Bridges and Hellyer, 1953). Methylbromide (MB) caused a significant reduction of ATP and arginine phosphoric acid (Arg-P) after a 1 min. exposure to the vapour and the insects were completely paralysed. The insects recovered temporarily in fresh air; also the levels of ATP and Arg-P. Lethal exposures to MB caused a slow but almost complete depletion of phosphoglycerate (PGA) after 1 hr. The fall in ATP was sometimes associated with an equivalent formation of AMP and free PO_4^{3-}, but in other cases the entire phosphorus of the ATP appeared as free PO_4^{3-}. This was not an artifact due to a non-uniform distribution of ^{32}P in the nucleotide phosphorus. After an exposure of 1 hr. to ethylenedibromide (EDB) vapour, when the insects had become motionless, the levels of ATP, Arg-P and G-6-P were unaltered, but there was a marked depletion of PGA with a corresponding rise of free PO_4^{3-}. Ethylenedichloride (EDC) vapour induced deep narcosis within 5 min. At this stage there was only a very slight fall in the levels of ATP and Arg-P and the

insects recovered completely in fresh air. *cyclo*-Propane similarly caused deep narcosis with little effect on the phosphorus distribution. Lethal exposure to EDC for 1 hr., unlike *cyclo*propane, caused a considerable depletion of ATP and Arg-P. The PGA level was unaffected by EDC. The slow depletion of PGA by MB and EDB suggests a common inhibition of triose phosphate dehydrogenase and therefore resembles iodoacetate in this respect (Cori, Slein & Cori, 1948). The spectacular depletion of ATP and Arg-P by MB suggests a rapid blocking of the phosphorylation of nucleotide acceptors these possibly then being lost to alternative metabolic pathways.

The delayed depletion of ATP by EDC supports the view of Johnson & Quastel (1953) that narcotics 'impede the oxidative synthesis' of ATP. The immediate narcosis induced by EDC and the extended narcosis induced by *cyclo*propane without significant depletion of ATP do not, however, support their view.

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The Use of ^{14}C-Algal Protein as a Labelling Agent for Determining the Life Span of Erythrocytes.

By R. S. Piha.* (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

For the determination of the life span of erythrocytes the isotope content of the haemin portion of the haemoglobin has been measured in experiments carried out by using as labelling agents [^{15}N]- or [^{14}C]-glycine (Shemin & Rittenberg, 1946; Neuberger & Niven, 1951; James, Abbot, Brooks & Evans, 1954; Berlin, Meyer & Lazarus, 1951) and deuterium-labelled acetate (Ponticorvo, Rittenberg & Bloch, 1949), while for the same purpose the globin part has been isolated after administration of carboxyl-labelled glycine (Grinstein, Kamen & Moore, 1949) and DL-lysine labelled with ^{14}C (Bale, Yuile, DeLaVergne, Miller & Whipple, 1949).

In this work the average life span of rabbit erythrocytes has been determined by measuring the isotope content of globin after injecting ^{14}C-labelled amino acids obtained from algal protein. A hydrolysate from 41 μc of protein (12·5 μc/kg.) was administered intravenously by a single injection. The specific activity of globin isolated from the circulating erythrocytes was determined by the method of Bradley, Holloway & McFarlane (1954) at intervals over a period of 70 to 80 days. The method of Strumia & Sample (1951) was adapted for the preparation of globin from small volumes of red cells (Dovey, Holloway, Piha, Humphrey & McFarlane, 1954).

The isotope content of the normal rabbit globin increases progressively, reaching a plateau at the fifth day. It remains constant for about 30 days after which it begins to decrease steadily, the falling part of the time curve representing the death rate of labelled cells. An average value of about 52 days was found for the life span of normal rabbit erythrocytes, i.e. about 15 days shorter than that found by Neuberger & Niven (1951) and 10 days longer than that reported by Seno, Kawai, Kanda & Nishikawa (1953) using a method based on the maturation of reticulocytes. The results agree fairly closely with those obtained by Burwell, Brickley & Finch (1953) using ^{55}Fe-tagged erythrocytes. The life span of rabbit erythrocytes has been estimated in different physiological states, and found to vary between 44 and 53 days.

The advantages of using algal protein hydrolysates for determining the life span of erythrocytes in which all the carbon atoms of the different amino acids are labelled (Catch, 1954) will be discussed.

The plateau found in the specific activity of normal globin and its steady fall support the view that the haemoglobin of red cells is metabolically inactive during the life span of the red cells.

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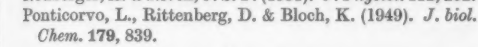
Observations on the Structure of Bacitracin *A*. By I. M. Lockhart and E. P. Abraham. (*Sir William Dunn School of Pathology, University of Oxford*)

The sequence of amino acids in bacitracin *A* has been established in two laboratories (Lockhart, Newton & Abraham, 1954; Craig, Hausmann & Weisiger, 1954; Lockhart & Abraham, 1954):



All three functional groups of the lysine residue are bound, forming a branched chain. Further information has now been obtained about this part of the molecule.

The bonds joining lysine to isoleucine and one of the aspartic acid residues are particularly stable. When bacitracin *A* was treated with 11 N-HCl at 80° for 43 hr., no free lysine was observed although the other amino acids were present in large amounts. The small number of peptides remaining were readily separated by chromatography and ionophoresis on paper and shown to be Asp.Lys, Ileu.Lys, Asp.-(Ileu).Lys and Ileu.Phe. Reaction of the first two peptides with FDNB and subsequent hydrolysis yielded α-DNP-lysine and ε-DNP-lysine respectively, which were distinguished by ionophoresis on paper in sodium borate buffer, pH 9·1. The α-amino group of lysine therefore appears to be joined to an isoleucine residue and the ε-amino group to an aspartic acid residue.

The second aspartic acid in bacitracin *A* is readily liberated on acid hydrolysis. Hence, a fraction which contained preponderantly this aspartic acid could be separated from the aspartic acid linked to lysine. The behaviour of the two fractions towards D-aspartic acid oxidase (Still, Buell, Knox & Green, 1949) was tested in the Warburg apparatus. Considerable racemization appeared to have occurred and the results were not clear-cut. However, they favoured the view that the aspartic acid linked to lysine had the L- configuration and the second aspartic acid the D- configuration.

One of the isoleucine residues in bacitracin *A* appears to be D-*allo*isoleucine (Piez, 1954). It has been found that if bacitracin *A* is deaminated with nitrous acid before complete hydrolysis the hydrolysate no longer shows the presence of *allo*isoleucine on columns of Dowex 50 (Moore & Stein, 1951). Partial hydrolysates of the deaminated material still contain the peptides Asp.Lys, Ileu.Lys, Asp.-(Ileu).Lys, and have given evidence that the isoleucine joined to the amino group of phenylalanine still remains intact. It therefore seems probable that the *allo*isoleucine is next to the latent cysteine residue.

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Factors Affecting the Free Amino Acid Composition of Fresh and Iced Skeletal Muscle of North Sea Codling (*Gadus callarias*). By N. R. Jones. (*Department of Scientific and Industrial Research, Food Investigation Organization, Torry Research Station, Aberdeen*)

Codling muscle was homogenized immediately *post mortem* into 75% v/v ethanol and prepared for chromatographic evaluation as described previously (Jones, 1954*a*). Over the year, average free amino acid composition per 100 g. muscle was: taurine, 300 mg.; glycine, 21 mg.; alanine, 15 mg.; glutamic acid, 8·0 mg.; L-methylhistidine, 6·7 mg.; leucine, 7·1 mg.; valine, 3·5 mg.; β-alanine, 1·2 mg.; aspartic acid, 1·9 mg.; serine, 1·4 mg.; lysine, 1·9 mg.; cysteic acid, 1·2 mg.; histidine, trace (< 0·5 mg.); proline, trace; tyrosine, trace; phenylalanine, trace; threonine, trace. The basic amino acid picture agrees

roughly with the qualitative studies of Shewan (1953).

Seasonal variations are found in values for certain amino acids. Thus taurine has a peak value at 350–450 mg./100 g. muscle in May–July falling to 100–150 mg. in December–February. Lysine, below 0·5 mg./100 g. for most of the year, rises to 12–15 mg./100 g. in January–February, the spawning period. The extent to which such changes relate to differences in food supply, environmental temperature, sex and gonad maturation is uncertain. Greene (1919) has demonstrated apparent mobilization of protein constituents of *Onchorrhynchus tschawytscha* during gonad maturation. Budanova (1952) found increased muscle protein lysine in *Acipenser acipenser* at the same period.

The free amino acid composition of the muscle of gutted codling alters considerably during storage in ice under fish-trade conditions. Early changes (0–4 days) appear to derive primarily from leaching by ice-melt water, 'drip' and the actions of autolytic enzymes. Later changes result from the additional effects of spoilage micro-organisms. At ambient temperature 2·5°, contents of 1-methylhistidine and β-alanine, liberated initially by muscle anserinase (Jones, 1954*b*), increase for 8–10 days to 60–80 and 40–50 mg./100 g. muscle respectively. Thereafter they fall because of increasing relative activities of other agencies. Taurine, relatively stable to microbial attack, is progressively leached. Lysine increases throughout storage to a value of 40–60 mg./100 g. after 15 days in ice. After a short initial decrease, values for most other amino acids (e.g. glycine) increase for 7–15 days apparently from microbial activity and then fall again.

At ambient temperature 0° contents of free amino acids were higher than at 2·5°, possibly because of lowered leaching losses. The position is somewhat complicated by the recent finding (Shewan, personal communication) that lowered ambient temperature raises the bacterial count of iced fish.

Incubation of aerated mixed fresh muscle and skin suspensions at 0° leads to development of a typical marine spoilage microflora and accumulation of free glutamic and aspartic acids, alanine, β-alanine, lysine, glycine, valine, leucine and 1-methylhistidine. However, non-removal of bacterial metabolites and by-products by leaching may limit the value of such experiments.

This work was carried out as part of the programme of the Food Investigation Organization of the DSIR.

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Some Chemical Properties of a Naturally occurring Antagonist of Dihydrostreptomycin. By J. W. Cornforth and A. T. James. (*National Institute for Medical Research, London, N.W. 7*)

The product isolated from the culture medium (Lightbown, 1954) formed crystalline plates, m.p. variable in different specimens between 136 and 148°. It dissolved in warm sodium carbonate solution and was reprecipitated by acid. The simplest formula agreeing with the elementary analyses was $C_{17}H_{23}O_2N$.

Oxidation with cold dilute potassium permanganate gave, together with unidentified acidic material, a steam-volatile acidic product which was shown by gas-liquid chromatography (James & Martin, 1952) to be made up almost entirely of *n*-heptoic and *n*-octoic acids in the approximate ratio of 1:3.

Reduction with zinc in acetic acid led in excellent yield to a crystalline product, m.p. 122–124°, which was shown by analysis to have one oxygen atom less than starting material. This substance resembled in many ways the 'Pyo' compounds of Hays *et al.* (1945). In particular the ultraviolet absorption spectrum of the reduction product was indistinguishable from that of 2-heptyl-4-hydroxyquinoline ('Pyo 1*b*') and the mixed melting-points with synthetic 2-heptyl- (m.p. 143°) or 2-octyl-4-hydroxyquinoline were intermediate.

When the reduction product was run on a partition chromatogram using ethylene glycol as the stationary phase and benzene–20% *cyclo*hexane as mobile phase, four substances were shown to be present. Comparison with the behaviour of synthetic 2-heptyl- and 2-octyl-4-hydroxyquinoline on the chromatogram suggested that both were present in the reduction product.

The absence of significant amounts of higher fatty acids than C_8 from the oxidation products suggests that one of the peaks on the chromatogram is due to a hydroxyquinoline with an unsaturated side chain. In agreement with this, small amounts of aldehydic material have been obtained by ozonolysis of the antagonist. The second peak on the chromatogram occupies the position to be expected from a hydroxyquinoline with a C_9 side chain. Tentatively, we suggest that 2-(2-nonenyl)-4-hydroxyquinoline is present. This hypothesis explains the heptoic acid

found on oxidation; moreover no double bond conjugated with the ring can be present as this would affect the ultraviolet absorption. It also explains the relative instability of the antagonist to atmospheric oxidation; the α-methylene group would be expected to be highly reactive. The fourth component shown by the chromatogram may well be due to traces of a 4-hydroxyquinoline with a side chain longer than C_9.

The evidence suggests strongly that the antagonist is a mixture of 4-hydroxyquinoline-*N*-oxides having the side chains mentioned above. 2-heptyl-4-hydroxyquinoline-*N*-oxide has been synthesized and its properties compared with those of the antagonist.

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Inhibition of Cytochrome System of Heart Muscle and of *Staphylococcus aureus* by 2-Heptyl-4-hydroxyquinoline-*N*-oxide, an Antagonist of Dihydrostreptomycin. By J. W. Lightbown and F. L. Jackson (introduced by A. T. James). (*National Institute for Medical Research, London, N.W. 7 and King's College Hospital Medical School, London*)

Synthetic 2-heptyl-4-hydroxyquinoline-*N*-oxide prepared by Cornforth & James (1954) would antagonize approximately 200 times its weight of dihydrostreptomycin, being three times less active than the natural antagonist mixture isolated by Lightbown (1954) from *Pseudomonas pyocyarea* cultures. Since the heptyl compound is the major component of the natural mixture, one or more of the other components must have a considerably higher activity. Like the natural mixture, concentrations of 2-heptyl-4-hydroxyquinoline-*N*-oxide higher than those necessary for antagonism inhibited growth of *Bacillus pumilus* and *Staphylococcus aureus*.

Manometric experiments with *Staph. aureus* suspensions in the presence of succinate showed that O_2 uptake was 60% inhibited by 0·35 μg. of 2-heptyl-4-hydroxyquinoline-*N*-oxide/ml., but with glucose as substrate ten times as much was required to produce a comparable effect. Intravenous injection into mice of 10 mg. of 2-heptyl-4-hydroxyquinoline-*N*-oxide/kg. body weight produced rapid cessation of respiration and death.

Microspectroscopic examinations showed that 2-heptyl-4-hydroxyquinoline-*N*-oxide inhibited oxidation of reduced cytochrome *b* in heart muscle succinic oxidase system, and of the *b* and *b*, components of *Staph. aureus* and *Bacillus subtilis* respectively. Oxidation of the other cytochrome components was unaffected. With succinate as substrate, O_2 uptake by pig heart succinic oxidase system was 50% inhibited by 0·35 μg. of 2-heptyl-4-hydroxyquinoline-*N*-oxide/ml., but oxidation of *p*-phenylenediamine by the same system was unaffected by 7 μg./ml. This suggests that 2-heptyl-4-hydroxyquinoline-*N*-oxide has no significant effect on cytochromes *c*, *a* or cytochrome oxidase, these manometric findings are consistent with the spectroscopic observations. When measured by the Thunberg technique, succinic dehydrogenase activity of heart muscle preparations and of suspensions of *Staph. aureus* was not inhibited by 2-heptyl-4-hydroxyquinoline-*N*-oxide. Similar results have been obtained in these experiments using the natural antagonist mixture.

These results suggest that 2-heptyl-4-quinoline-*N*-oxide inhibits terminal respiration somewhere between succinic dehydrogenase and cytochrome *c* in the heart muscle preparation. Although *Staph. aureus* in contrast to heart muscle, appears to have no cytochrome *c*, the *b* components although different spectroscopically appear to behave similarly in the presence of 2-heptyl-4-hydroxyquinoline-*N*-oxide, suggesting some sensitive step common to both systems.

The possible relationship of this action of 2-heptyl-4-hydroxyquinoline-*N*-oxide to the antagonism of streptomycin remains to be investigated.

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Location of Tritium in Stearic Acid Produced by Catalytic Tritiation of Elaidic Acid. By R. F. Glascock and L. R. R. Reinius.* (*National Institute for Research in Dairying, University of Reading*)

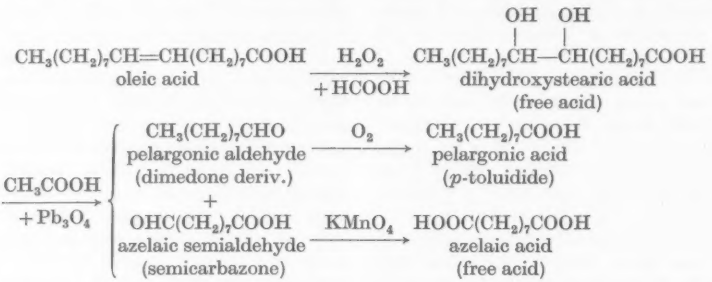
Work was recently reported from this laboratory (Duncombe & Glascock, 1954) in which stearic acid, prepared by the tritiation of oleic acid over a palladium catalyst, was used. It was thought that this procedure would yield predominantly 9:10-[^{3}H] stearic acid but some of the biological results obtained when the product was used indicated that this should be confirmed before proceeding with further work.

After feeding the [^{3}H]stearic acid to a lactating goat the unsaturated fatty acid fraction of the milk had been found radioactive suggesting that stearic acid had been biologically converted to oleic acid. Because it is much easier to degrade oleic than stearic acid advantage has been taken of this finding. [^{3}H]stearic acid, prepared from elaidic acid, was used and the unsaturated fatty acid fractions isolated from milk collected 23 and 31 hr. after dosing.

Carrier oleic acid was added to each sample and the mixtures then subjected to the chemical procedure shown on p. 17, the compounds being isolated as the derivatives indicated.

The dihydroxystearic acid was found to be highly radioactive confirming that conversion of stearic into oleic acid occurs in the goat. A comparison of the specific activities of the dihydroxystearic acid, the pelargonic aldehyde and azelaic semialdehyde obtained from both samples, showed that no loss of activity had occurred during fission of the dihydroxystearic acid. It is of interest to note that the total activity in the pelargonic aldehyde was 1·7 times that in the azelaic semialdehyde.

Oxidation of the aldehydes to acids results in labilization of hydrogen originally attached to the C_9 and C_{10} positions of the parent oleic acid. It was found that the specific activity of the azelaic acid was an average of 8 % lower, and that of the pelargonic acid 20 % lower than that of the aldehydes. These values, being obtained by difference, are not as reliable as directly determined specific activities but they indicate that only about 3 % of the tritium in the original oleic acid resided in the $C_{(9)}$ position and about 12 % in the $C_{(10)}$ position. The assumption made in the communication cited that tritiation of oleic and elaidic acids yields a specifically labelled stearic acid is therefore wrong.



Thanks are due to Dr S. J. Folley, F.R.S., for making available the experimental animal and to Dr A. T. Cowie for administering the labelled material.

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* British Council Scholar, from the Veterinary College Helsinki.

DEMONSTRATIONS

Zone Electrophoresis of Pathological Sera Labelled with ^{131}I. By A. H. Gordon and A. S. McFarlane. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Comparative advantages of potato starch and glass powder as stablizing media have been examined.

Techniques for localization of bands by 'printing off' on to filter paper have been used. Subsequently such strips have been coloured with Durrum's reagent to show up bands of protein and passed through an automatic counter to record the ^{131}I.

Demonstrations of the Use of Salicylic Acid Hydrazide and of Laboratory Apparatus. By B. CAMBER (introduced by J. PATTERSON). (*Charing Cross Hospital, London, W.C.* 2)

The demonstrations include: (*a*) Salicylic acid hydrazide: a microchemical reagent for visual differentiation of aldehydes and ketones by fluorescence. (*b*) Salicylic acid hydrazone of pentanedione: a reagent for zinc. (*c*) Stabilized *p*-aminodiethylaniline and periodic acid as a sensitive reagent system for phenolic substances. (*d*) A type of separatory funnel incorporating a reaction flask stopper. (*e*) A shaking device suitable for separatory funnels using a spring-mounted multiple holder. (*f*) A washer for laboratory glassware incorporating side-valve action, simple interchangeable components and automatic off-on action. (*g*) A modified all-glass pipette-washer ensuring adequate drainage between each filling-emptying cycle. (*h*) Tissue-washers for use with (*g*) permitting simultaneous multiple washing. (*i*) A design of syringe which can be charged by pipette to ensure accurate dosage of drugs.

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

The *in vitro* protection of cholinesterases against some organophosphorus inhibitors. By W. K. Berry, K. P. Fellowes, P. J. Fraser, J. P. Rutland and A. Todrick

The role of glycerylphosphorylcholine and glycerylphosphorylethanolamine in liver phospholipid metabolism. By R. M. C. Dawson

The sulphatase of ox liver. 4. A note on the inhibition of sulphatases *A* and *B*. By A. B. Roy

Mode of insecticidal action studied with labelled systems. Phosphorylated compounds in the muscle of the adult housefly, *Musca domestica*, L. By F. P. W. Winteringham, Patricia M. Bridges and G. C. Hellyer

The biological action of substances related to thyroxine. 10. The deiodination of thyroxine in thyroidectomized rats. By J. H. Wilkinson and A. J. Feetham

The hydration of the cornea. By H. Davson

Studies on sulphatases. 9. The arylsulphatases of mammalian livers. By K. S. Dodgson, B. Spencer and J. Thomas

Fate of porphobilinogen, administered enterally or parenterally, in the rat. By A. Goldberg

The free energy change associated with the hydrolysis of the thiol ester bond of acetyl coenzyme A. By K. Burton

The distribution of diphtheria antitoxin in pepsin-digested horse antiserum. By C. G. Anderson

The effects of salicylate and adrenocortical preparations added *in vitro* upon the glycogen content of liver slices. By M. J. H. Smith

The action of polylysine on the conversion of fibrinogen into fibrin by coagulase thrombin. By Naomi Biezunski, E. Shafrir, A. de Vries and E. Katchalski

Glucuronide metabolism in plants. 2. The isolation of flavone glucosiduronic acids from plants. By C. A. Marsh

The amino acid composition of wool. By M. C. Corfield and A. Robson

Purification and properties of some enzymes in human seminal plasma. By F. Lundquist, Th. Thorsteinsson and O. Buus

The effect of *o*-phenylenediamine on the biosynthesis of vitamin B_{12}: a new vitamin B_{12} analogue. By K. H. Fantes and Cynthia H. O'Callaghan

The vitamin B_{12} group. Presence of 2-methyl purines in factors A and H and isolation of new factors. By F. B. Brown, J. C. Cain, Dorothy E. Gant, L. F. J. Parker and E. Lester Smith

The biosynthesis of vitamin B_{12}-like compounds. By J. E. Ford, E. S. Holdsworth and S. K. Kon

The application of the ceric sulphate-arsenious acid reaction to the detection of thyroxine and related substances. By C. H. Bowden, N. F. Maclagan and J. H. Wilkinson

The inhibition of horse-liver esterase by rhodamine B. By Jane Burch

The oxidation of indolyl-3-acetic acid by waxpod bean root sap and peroxidase systems. By R. H. Kenten

Studies on rhodopsin:

8. Retinylidenemethylamine, an indicator yellow analogue. By G. A. J. Pitt, F. D. Collins, R. A. Morton and Pauline Stok

9. pH and the hydrolysis of indicator yellow. By R. A. Morton and G. A. J. Pitt

A modified Conway unit for microdiffusion analysis. By K. J. Öbrink

The isolation of 16-*epi*oestriol from the urine of pregnant women. By G. F. Marrian and W. S. Bauld

[P.T.O.

The content and distribution of cytochrome *c* in the fatty liver of rats. By M. U. Dianzani and I. Viti

The *N*-terminal residues of bovine plasma clot. By W. R. Middlebrook

The determination of ^{65}Zn in tissues with Geiger and scintillation counters. By T. E. Banks, R. Tupper, R. W. E. Watts and A. Wormall

Studies in detoxication:

60. The metabolism of alkylbenzenes. *iso*-Propylbenzene (cumene) and derivatives of hydratropic acid. By D. Robinson, J. N. Smith and R. T. Williams

61. The metabolism of alkylbenzenes. *tert.*-Butylbenzene. By D. Robinson and R. T. Williams

Kinetic studies on the metabolism of foreign organic compounds. 6. Reactions of some nuclear substituted benzoic acids, benzamides and toluenes in the rabbit. By H. G. Bray, Brenda G. Humphris, W. V. Thorpe, K. White and P. B. Wood

The hydrolysis of *n*-hexanamide by rabbit-liver extracts. By H. G. Bray, K. White and P. B. Wood

Interference by amino acids in the estimation of sugars by reductometric methods. By R. E. Strange, F. A. Dark and A. G. Ness

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